Regulation of ERK1/2 signaling in melanoma

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Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Arbeit von mir selbständig und unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde. Weiterhin habe ich noch keinen Promotionsversuch unternommen, oder diese Dissertation in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt

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1. Abstract

The mechanisms that enable cells to regulate their gene expression and thus their metabolism, proliferation or cellular behaviour are not only important to understand the basic biology of a living cell, but are also of crucial interest in cancerogenesis. Highly interwoven and tightly regulated pathways are the basis of a robust but also flexible regulatory network. Interference with these pathways can be either causative for tumorigenesis or can modify its outcome. The receptor tyrosine kinase (RTK) and RAS dependent pathways leading to AKT or ERK1/2 activation are of particular interest in melanoma. These signaling modules are commonly activated by different mutations that can be found in various pathway components like NRAS, BRAF or PTEN.

The first part of this work deals with the diverse and versatile functions of the ERK1/2 pathway feedbackregulator MKP2 in different cellular, melanoma relevant settings. In addition, a functional role of the AP1-complex member FOSL1, an ERK1/2 transcriptional target being implicated in the regulation of proliferation, is demonstrated.

Secondly, aspects of direct pharmacological inhibition of the ERK1/2 pathway with regard to the induction of apoptosis have been analysed. Due to the high frequency of melanoma related mutations occurring in the RAS/RAF/MEK/ERK pathway (e.g. $NRAS^{Q61K}$, $BRAF^{V600E}$), inhibition of this signaling cascade is deemed to be a promising therapeutic strategy for the treatment of malignant melanoma. However, although in clinical trials mono-therapeutic treatment with MEK- or RAF inhibitors was successful in the short run, it failed to show satisfactory long-lasting effects. Hence, combination therapies using a MAPK pathway inhibitor and an additional therapy are currently under investigation. I was able to demonstrate that inhibition of MEK using the highly specific inhibitor PD184352 can have a protective effect on melanoma cells with regard to their susceptibility towards the apoptosis inducing agent cisplatin. Single application of cisplatin led to strong DNA damage and the induction of caspase-dependent apoptosis. Additional administration of the MEK inhibitor, however, strongly reduced the apoptosis inducing effect of cisplatin in several melanoma cell lines, These cells displayed an increased activation of the serine/threonine kinase AKT after MEK inhibition. This AKT activation concomitantly led to the phosphorylation of FOXO transcription factors, attenuating the cisplatin

induced expression of the BH3-only protein PUMA. PUMA in turn was important to mediate the apoptosis machinery after cisplatin treatment. My results also indicate a participation of RTKs, in particular EGFR, in mediating MEK inhibitor induced activation of AKT.

These results demonstrate that inhibition of the RAS/RAF/MEK/ERK signaling pathway in melanoma cell lines does not necessilary have favourable effects in a cytotoxic co-treatment situation. Instead, it can even enhance melanoma survival under pro-apoptotic conditions.

2. Zusammenfassung

Die Mechanismen in einer Zelle, die die Genexpression und somit den Stoffwechsel, das Wachstum und das gesamte Zellverhalten steuern, sind ebenso bedeutsam für das Verständnis der grundlegenden Biologie einer lebenden Zelle wie für die Vorgänge der Krebsentstehung. Dabei bilden hochvernetzte, und strikt regulierte Signaltransduktionswege die Basis für ein belastbares und zugleich hochflexibles regulatorisches Netzwerk. Die Störung solcher Signalkaskaden kann zum einen ursächlich aber auch modifizierend auf die Bildung von Tumoren wirken. Die von Rezeptortyrosinkinasen (RTK) und RAS abhängigen Signalwege, die zur Aktivierung von AKT und ERK1/2 führen, sind hierbei von besonderem Interesse für die Entstehung des malignen Melanoms. Mutationen in Komponenten dieser Wege (z.B. NRAS, BRAF oder PTEN), die die Signalstärke erhöhen kommen in Melanomen sehr häufig vor.

Im ersten Teil dieser Arbeit wurden die unterschiedlichen und vielfältigen Funktionen von MKP2, einem Feedbackregulator des ERK1/2-Weges, unter verschiedenen zellulären Rahmenbedingungen, untersucht. Des Weiteren wird eine Funktion des zum AP1-Komplex gehörenden FOSL1, einem unter transkriptioneller Kontrolle des ERK1/2-Weges stehendem Transkriptionsfaktors, hinsichtlich der Steuerung der Zell-Proliferation gezeigt.

Weiterhin habe ich Aspekte der direkten pharmakologischen Inhibition des ERK1/2-Weges hinsichtlich ihres Effekts auf die Auslösung von Apoptose untersucht. Aufgrund der Häufigkeit von Mutationen in Genen, die für Proteine des ERK1/2-Weges kodieren (z.B. $NRAS^{Q61K}$, $BRAF^{V600E}$), gilt die Inhibition dieses Signalwegs als vielversprechende Strategie zur Behandlung des Melanoms. Auch wenn klinische Studien, die Inhibitoren für MEK oder RAF als Einzelmedikamente verwenden, bei mehrmonatiger Behandlung sehr erfolgreich sind, konnten so keine langfristigen Erfolge erzielt werden. Aus diesem Grund werden nun Kombinationstherapien, die einen Inhibitor des ERK1/2-Weges und eine weitere Form der Therapie kombinieren, untersucht.

Der zweite Teil dieser Arbeit beschreibt, dass der spezifische MEK Inhibitor PD184352 Melanomzellen vor der Apoptosewirkung von Cisplatin schützen kann. Einzelbehandlung mit Cisplatin führt hierbei zur Akkumulation von DNA Schäden, die wiederum Caspase-abhängig Apoptose induzieren. Zusätzliche Anwendung des

MEK Inhibitors verringerte jedoch in einigen Zelllinien das Potential von Cisplatin, Apoptose auszulösen. Diese Zellen zeigten eine verstärkte Aktivierung der Serin/Threonin-KInase AKT nach MEK Inhibition. Diese AKT Aktivierung führte zur Inaktivierung der FOXO Transkriptionsfaktoren, was wiederum die Expression des pro-apoptotischen BH3-only Proteins PUMA verringerte. PUMA selbst ist ein wichtiger Bestandteil der Apoptose Maschinerie, die durch Cisplatin aktiviert wird. Die im Rahmen dieser Arbeit erhaltenen Befunde deuten darauf hin, dass RTKs, im besonderen EGFR, bei diesem Crosstalk eine Rolle spielen.

Diese Ergebnisse zeigen, dass die Inhibition des RAS/RAF/MEK/ERK Signalweges im Melanom nicht zwangsläufig von Vorteil sein muss, falls die Zellen gleichzeitig mit einem genotoxischen Medikament behandelt werden. Hier kann sie sogar die Überlebensfähigkeit von Melanomzellen unter Apoptose induzierenden Bedingungen verbessern.

3. Introduction

3.1. Melanoma

Skin cancers constitute the most common form of cancer in Germany. The majority are non-melanoma skin cancers like basal cell carcinoma and squamous cell carcinoma [1], representing non invasive and rarely fatal diseases. Melanoma represents with respect to its occurrence a rather rare form of skin cancer being causative for only 5% of cases in caucasians. However, it still accounts for about 75% of skin cancer related deaths. With an annual increase in incidence of about 3-5%, melanoma moreover constitutes the most common cancer in young adults (25-29 years) [2][3].

Melanoma is a malignant form of skin cancer that is derived from melanocytes. Melanocytes, which differentiate from neural crest progenitors, reside mainly in the skin either in the basal layer of the epidermis or in the hair follicles [4]. The main biological function of melanocytes is to produce melanin, the pigment that is responsible for the color of hair and skin. Melanin was described to play a role not only in thermoregulation, as an antibiotic, a cation chelator and free radical sink [5], but most importantly in protecting the different cells of the skin from UV-induced DNA damage and mutagenesis [6]. However, since melanin synthesis can cause superoxide radicals under persisting UV irradiation it also contributes to clearance of cells that were highly exposed to mutagenic irradiation [7]. Melanin production in melanocytes as well as its further distribution to keratinocytes is triggered by different UV-dependent mechanisms. Firstly, direct effects of UV on melanocytes [8] and, secondly α-MSH mediated MITF activation were found to be responsible for increased melanin production [9]. MITF is a transcription factor belonging to the family of basic helix-loop-helix leucine zipper factors. It is considered to be a melanocyte master regulator, which is not only involved in regulation of melanocyte specific genes required for melanin synthesis like *tyrosinase*, *tyrosinase related protein 1* or *dopachrome-tautomerase*, but has also more direct effects on melanocyte proliferation, invasion, differentiation, replication, DNA repair, and mitosis [10–12]. MITF is commonly found to be amplified in melanoma (20% of cases) [13] and contributes to the regulation between proliferative or invasive melanoma phenotypes [14].

Melanomagenesis, the progression from normal melanocytes to malignant melanoma can be divided into several distinct steps. Loss of growth control, which is commonly due to activating ERK1/2-pathway mutations leads to the first hyper-proliferative behavior resulting in the formation of benign nevi, which display characteristics of senescence and very rarely progress to advanced melanoma stages [15]. However, additional loss of tumor suppressors overcomes senescence and can lead to progression to later melanoma stages. The radial growth phase (RGP), where cells are propagating within the epidermis is considered to be the first stage of melanomagenesis. During the vertical growth phase (VGP), the next step of progression, the cells enter the dermis. Cells can now disseminate from the primary tumor and invade other tissues, thus introducing the metastatic form of the disease. However, melanomagenesis does not necessarily occur linear through the different stages. Single melanocytes or nevi can progress directly to RGP or VGP melanoma, and both intermediate stages can develop straightforward to the metastatic stage [4], [16].

3.2. Signaling pathways in melanoma

Due to different locations, morphologies, mutagenic stresses and a plethora of resulting mutations, there are many different subtypes of melanoma contributing to the often referred high heterogeneity in melanoma. Besides numerous mutations that occur in tumor suppressor genes like *p53*, *CDKN2A*, *RB1*, *CDK4* or *CCND1* a common feature of melanoma cells is the activation of the RAS/PI3K/AKT (in short: AKT) pathway and even more prevalent the RAS/RAF/ERK1/2 (in short: ERK1/2) pathway. An overview of the AKT and the ERK1/2 signaling pathways, containing for this work relevant features, is depicted in Figure 1.

3.2.1. ERK1/2 pathway

The ERK1/2 pathway, being the most thoroughly analyzed MAPK pathway, is of essential importance not only in the course of melanomagenesis. It constitutes a signaling cascade which relays extracellular stimuli to nuclear events employing a series of phosphorylation events. Growth factor activated receptor tyrosine kinases transmit their ligand dependent signaling to the membrane bound RAS protein which is activated in its GTP-bound state. This activation is mediated by SH2 domain containing adaptor proteins (e.g. GRB2) and guanine-nucleotide exchange factors (e.g. SOS). RAS then recruits RAF to the membrane where it gets stimulated. Activated RAF phosphorylates and activates MEK1 and MEK2 which in turn activate ERK1 and ERK2 by phosphorylating specific threonine and tyrosine residues (reviewed in [17]). Additional layers of ERK1/2 pathway regulation are achieved by other factors and the involvement of scaffolding proteins [18]. ERK then shuttles to the nucleus where it transmits the signal to downstream transcription factors like MYC, FOS, CREB and others.

In virtually all cases of sporadic melanoma, activating mutations render the ERK1/2 cascade stimulation independent and constitutively hyperactivated [19]. This activation is caused by different mutations. The most prevalent oncogenic mutation is the $BRAF^{V600E}$ mutation, which was found to be present in 60% of melanomas [20]. In 80% of cases the valine at codon 600 is changed to glutamic acid (V600E). However there are also changes for lysine (V600K) or very rarely aspartic acid or arginine (V600D/R) [21]. $BRAF^{V600E}$ is characterized by a conformational change in structure,

due to the phospho-mimetic glutamic acid, which is located between the Thr598 and Ser601 phosphorylation sites, two residues implicated in the activation of wildtype BRAF. The mutant form does no longer require phosphorylation of these sites to get activated. The amino acid change leads to an ~500 fold increased kinase activity [22]. Interestingly, only BRAF, but not ARAF or CRAF, is frequently activated by point mutations in cancer. This is due to the absence of a constitutive charge in the regulatory N- region of the BRAF kinase domain. ARAF and CRAF harbor this charge that prevents the V600E equivalent mutations in these isoforms from inducing the changes required for constitutive activation. Therefore an additional mutational event would be required to transfer ARAF or CRAF to a highly activated state [23]. However, CRAF plays an important role in melanoma signaling, because it is the preferred isoform in cells that are driven by oncogenic, mutated RAS [24]. Furthermore it has been shown that CRAF contributes to $BRAF^{V600E}$ driven melanomagenesis since it gets trans-activated by heterodimerizing with BRAF [25].

Other, rather common oncogenic drivers of ERK1/2 pathway proteins are RAS-family members. Most RAS-driven melanomas show an activating mutation in NRAS (Q61K/R) where a glutamine is exchanged for lysine or arginine. This mutation can be found in 15-30% of melanomas [4]. In very rare melanoma cases, however, HRAS but not KRAS was found in oncogenically hyperactivated variations [26]. Oncogenic mutations in RAS family members, which primarily occur at amino acids 12, 13 or 61, all interfere with the function of the intrinsic GTPase domain. After activation of RAS by SOS mediated exchange of GDP by GTP at the membrane, the mutated proteins cannot return to their inactive state, which would normally be achieved by hydrolysis of the bound GTP. Thus the oncogenic mutations keep RAS in a constitutively active conformation, resulting in the enhancement of downstream ERK1/2 signaling. BRAF and NRAS mutations are virtually mutually exclusive in carcinogenesis, hinting at their redundant function in ERK1/2 pathway activation [26]. Additionally, however, mutated RAS proteins are also capable of activating additional signaling mechanisms. Besides activation of RAL-GEF, PKC-ζ, and phospholipase-ε [27], the PI3K/AKT pathway is of high importance in this context [28]. While $BRAF^{V600E}$ seems to cooperate with PTEN loss, $NRAS^{Q61K}$ is again mutually exclusive to loss of this tumor suppressor, indicating the ability of RAS to activate this pathway as well [29].

Besides the above mentioned oncogenes, RTKs also drive the ERK1/2 pathway. As upstream components of signaling cascades, RTKs are putatively able to stimulate

even more signaling pathways than RAS. KIT is the only RTK which was identified to carry an activating mutation (L576P) in melanoma [30]. Mutated KIT was found to be present in 23% of cases at mucosal, acral, or cumulative sun-damaged skin sites [31]. Additionally, copy number alterations (quantitative), but no gain of function (qualitative) mutations of EGFR and MET have been described. However, these chromosomal amplifications seem to be very late events in melanomagenesis [26].
Quite recently mutated GNAQ and GNA11 have been identified as frequently occurring oncogenes in a specific subset of melanocytic lesions (blue nevi and uveal melanoma). Both proteins belong to the family of trimeric G-proteins (α-subunit) and are responsible for the transduction of G-protein coupled receptor signals to corresponding effectors. The oncogenic versions display defects in their GTPase domain, mechanistically comparable to $NRAS^{Q61K}$. Mediated by activation of PKC, oncogenic $GNAQ^{Q209L}$ was shown to activate ERK1/2 signaling [32].
Altogether, the ERK1/2 pathway is of central importance for melanomagenesis, contributing to increased proliferation, differentiation, survival, anchorage independent growth, metastasis, EMT induction and immune evasion [27], [33–35].

3.2.2. PI3K-AKT pathway in melanoma

Besides the ERK1/2 pathway, the AKT pathway is commonly deregulated in human melanoma as well as in other malignancies. Comparable to the ERK1/2 pathway, the AKT pathway transmits extracellular signals that are recognized by membrane bound receptors to intracellular effectors, using RAS as a possible signal mediator.
RAS recruits the catalytic subunit of PI3K (p110α, β, γ or δ) to the membrane where it gets into contact with the regulatory subunit (p85, p65, p55 or p101) and the substrate PIP2 (phosphatidylinositol-3,4-biphosphate). The lipid-kinase domain of PI3K then phosphorylates PIP2 to generate the second messenger PIP3 (phosphatidylinositol-3,4,5- triphosphate). This reaction is antagonized by PTEN. The membrane tethered PIP3 in turn stimulates PDK1 to phosphorylate AKT, which gets sequestered at the membrane and thus activated. Fully activated AKT requires an additional phosphorylation event, which can be mediated by mTOR (in complex with RICTOR) [36], or AKT auto-phosphorylation [37], amongst others. AKT subsequently phosphorylates a variety of intracellular effector molecules, e.g. BAD, RAC, FOXO1/3a, GSK3 and mTOR (reviewed in [38] [39]).

Similarly to the ERK1/2 pathway, activation of AKT signaling is a common event in melanoma. Besides the already mentioned RAS mutations other pathway stimulating alterations have also been reported. *PIK3CA* encoding the catalytic PI3K subunit p110α was found to be mutated in a variety of different malignancies. In melanoma, these mutations occur with an incidence of 3% [40]. More common is the loss of the tumor suppressor PTEN. PTEN protein expression levels were described to be lost or at least strongly reduced in 19% of melanomas [41]. Loss of PTEN strongly enhances the levels of PIP3, leading to elevated levels of phosphorylated AKT. AKT3, the predominant melanoma isoform of AKT [42] was found to be very rarely mutated but instead often amplified in melanoma [75] [76]. All these oncogenic loss and gain of functions of AKT pathway proteins lead to the pathway`s hyper-activation in up to 60% of sporadic melanomas [26].

Elevated AKT signaling output was shown to have multiple advantages for melanoma cells with regard to diverse oncogenic properties, mediated by more than 100 described AKT phosphorylation targets [45]. Most importantly, AKT signaling is implicated in cell cycle control and thus proliferation by regulating p21, p27, mTOR or TSC2 [46]. However, mTOR as well as the AKT substrate GSK3 also play major roles in regulating cellular metabolism and protein synthesis [47]. Additionally increased survival and resistance to apoptosis is also linked to constitutively elevated AKT activity. This is mediated by direct phosphorylation of BAD or caspase9 and by sequestration of FOXO to the cytoplasm [48]. Finally, regulation of migration and invasion is also influenced by AKT, in an epithelial-mesenchymal transition (EMT) resembling manner [49]. EMT, a process firstly described to occur naturally during embryogenesis, describes the loss of E-cadherin mediated cell adhesion resulting in the loss of epithelial morphology and the gain of a mesenchymal phenotype with an increase in cell motility. Although the process of EMT describes events in epithelial cells, features of a comparable mechanism have been described in melanoma [33].

3.2.3. The model RTKs Xmrk and HERmrk as tools for analyzing oncogenic signaling in melanoma

To be able to analyze the different functions and features of signaling networks in oncogenic situations, it is important to use clearly defined signaling systems.

Additionally, it is of utmost importance to use models that resemble signaling features of actual human malignancies.

The first ever established animal model for melanoma was the Xmrk melanoma model in the *Xiphophorus* fish, which has been described already in the late 1920s [50], [51]. Here, melanoma can be induced by natural crossings of two different *Xiphophorus* species leading to hybrids that express the constitutively active EGFR orthologue Xmrk. These fishes develop melanomas with an incidence of 100%. (reviewed in [52]). When MITF-driven Xmrk was introduced into medaka fish, a well-established model organism comparable to zebrafish, it also induced melanoma formation [53]. Additionally, to be able to analyze the Xmrk induced signaling network as well as its consequences on a molecular level, the murine melanocyte cell line melan-a was engineered to express a chimeric version of Xmrk and EGFR called HERmrk. HERmrk thereby comprises the intracellular domain of Xmrk and the extracellular domain of the human EGFR. This allows triggering of Xmrk signaling by stimulation of the cells with human EGF. To test the effect of distinct signal strengths, clonal cell lines with different *HERmrk* expression levels were established. Among these are a high HERmrk expressing cell line (HERmrk^{hi}) and a cell line expressing intermediate levels of HERmrk (HERmrk^{me}) [54]. The main advantage of the Xmrk induced melanoma model is its independence from additional cooperating factors since Xmrk was shown to be sufficient to induce tumor formation [79]. Furthermore, activation of ERK1/2- and AKT-signaling, the major hallmarks of melanoma signaling, are characteristics for Xmrk induced melanoma formation [52]. Recently, the melan-a/ HERmrk system was used for a transcriptome study to identify transcriptional targets of Xmrk in the murine melanocyte cell line [55]. A part of the work described in this thesis deals with the validation of two different Xmrk targets, *Fosl1* and *Mkp2,* which were identified in this screen. FOSL1 is a component of the AP1 transcription complex constituting a known ERK1/2 downstream target. For another member of the AP1 complex, JUN, an important role in melanomagenesis has already been described [56]. MKP2 on the other hand is a MAPK feedback regulator attenuating MAPK signaling output.

3.3. Feedback regulation of the ERK1/2 pathway

The tight regulations of signal transduction pathways is of essential importance to allow normal cell maintenance and directed developmental processes and to prevent cellular transformation [57]. The actual output of signaling cascades is not only determined by an intrinsic or extrinsic stimulus but it is also altered on several additional levels within a specific cell population. Especially the control of signal strength, frequency as well as termination of a stimulating signal is indispensable for normal cell homeostasis [58].

The ERK1/2 pathway is negatively regulated by several different mechanisms. Besides a selective inhibition of nuclear targets by cytoplasmic sequestration as mediated by SEF [59], RKIP is a widely described inhibitor of ERK1/2 signaling, that is also deregulated in melanoma [60]. However, bona fide feedback inhibitors that are actually transcriptionally induced by ERK1/2 signaling are MAPK Phosphatases (MKPs) and SPROUTY/SPRED family members.

3.3.1. MAPK Phosphatases

MAPK phosphatases are members of the dual specificity phosphatase (DUSP) family. They regulate the activity of the MAPK pathway by removing the activating phosphates at specific tyrosine and threonine residues within the different MAPKs and thus decrease the pathway`s activity. They are thereby antagonizing the stimulating activity of MAPK kinases like MEK1/2. There are 10 different members within the MKP family with different substrate specificities for one or more of the different MAPKs (ERK1/2, JNK, or p38) [61]. All share a common structure: A C-terminal phosphatase domain, a non-catalytic N-terminal domain that comprises a MAPK recognition domain known as the kinase interaction motif (KIM), and sequences which determine the subcellular localization of the specific MKP. Substrate specificity and cellular localization divide the MKP family in three distinct subclasses. Class III MKPs (e.g. MKP5) being located in the nucleus or cytoplasm target only p38 and JNK, class II proteins (e.g. MKP3) target just ERK1/2 and are located in the cytoplasm, while class I proteins (e.g. MKP1 and MKP2) have a low substrate specificity and are located in the nucleus [62]. These diverse interaction abilities, coupled with the transcriptional regulation of MKP expression in response to

MAPK signaling, predicts a complex regulatory network, linking and integrating the different MAPK pathway branches [63]. Furthermore a certain redundancy also arises from this complex interaction network. However, distinct functions for several MKPs have also been described, especially regarding developmental aspects.

MKP3 for example is deregulated in several malignancies. An oncogene induced upregulation of MKP3 expression can be observed in myeloma, glioma, or breast cancer [64–66]. In melanoma, MKP3 has been shown to be upregulated after oncogenic BRAF and NRAS signaling [67] [68]. Interestingly, there is recent evidence that in case of melanoma MKP3 can have different functions. In a system of murine melanocytic cells that are characterized by low ERK1/2 signaling output and high MKP3 levels, additional overexpression of MKP3 increased anchorage independent growth and invasiveness. In contrast to this, in human melanoma samples overexpression of MKP3 led to reduced tumorigenicity [69].

The expression levels of MKP2 (DUSP4) has been shown to be altered activation of HERmrk signaling [55]. MKP2, a member of the class I MKPs, is also implicated in several different tumorigenic alterations. In rectal tumors as well as Xmrk induced melanomas an upregulation of MKP2 as consequence of oncogenic activation of ERK1/2 signaling has been observed [108] [55]. However, comparable to the diverse function of MKP3 in different tumor settings, MKP2 has also been described to be downregulated in various malignancies, like lung cancer or certain ovarian tumors [109] [110]. Additionally a non-redundant function for MKP2 in MEFs was described recently. MEFs derived from MKP2 knockout animals showed a strongly reduced proliferation with a concomitant block in G2/M transition and a higher rate of apoptosis after stimulation of JNK dependent signaling [71]. Interestingly, MKP2 was also identified to be a transcriptional p53 target under oxidative stress conditions and to be implicated in p53 induced apoptosis [73].

In the course of this work the role of MKP2 in melanoma was further analyzed, since it was discovered to be strongly induced in the HERmrk melanocyte model.

3.3.2. SPROUTY and SPRED proteins

The Sprouty family of proteins consists of four SPROUTY proteins (SPRY1-4) and three related SPRED proteins (SPRED1-3) [74]. Besides the MKPs they form a

second group of negative feedback regulators of MAPK signaling, antagonizing the activity of various RTKs like EGFR, FGFR, PDGFR, VEGFR and others.

The SPROUTY proteins have been analyzed much more extensively than the SPRED proteins. They all contain C-terminal cysteine rich sprouty domain (SPR) but differ within the N-terminal region that contributes to their functional divergence [75]. During development and organogenesis different family members seem to carry out various functions [74–76]. Although they can compensate in part for the loss of other family members, SPRYs are not able to completely rescue specific loss of functions as seen in the different phenotypes of *Spry2* and *Spry4* knockout mice and the embryonic lethality of double knockouts [77]. However, although the function of Sprouty proteins as RTK signaling antagonists is well established, the actual mode of action seems to be rather diverse and versatile and still remains a matter of debate.

Early studies showed that SPRY4 can interact with CRAF, thus executing its function even upstream of MEK [78]. Nevertheless, several different additional modes of action for Sprouty family members were also described. SPRY1 and 2 were shown to be able to prevent RAS activation downstream of GRB2/SOS [79]. However, other studies propose an interaction with a GRB2 homologue, that blocks MAPK signaling just downstream of the receptor. This was proposed for the unique SPRY homologue and Drk, the GRB2 homologue in *Drosophila* [80], and also for the mammalian SPRY1 and 2 [81]. Even further upstream, SPRY2 overexpression was shown to induce the secretion of a soluble factor that in turn was able to interfere with FGFR but not VEGFR mediated proliferation [82]. Also additional signaling pathways like G-coupled receptor signaling leading to activation of the phosphatidylinositol-specific phospholipase Cγ (PLCγ) are negatively regulated by SPRY proteins. SPRY1 and 2 were both described to be implicated in the reduction of PLCγ dependent second messengers like inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [83].

In terms of carcinogenesis, consistent with a role for SPRY2 in inhibiting important signaling pathways as well as proliferation, SPRY2 protein levels are decreased in hepatocellular and lung carcinomas [122] [123]. In breast and prostate carcinomas SPRY1, SPRY2 and SPRY4 were found to be downregulated as well [86–88]. In contrast, for melanoma a different situation has been described. In cell lines carrying an oncogenic BRAF or NRAS allele, levels of *SPRY2* were strongly enhanced [67]. However, a functional effect as a growth suppressor has been described only in the case of melanocytes or BRAF wild type melanomas where decreased SPRY2 levels

enhance ERK1/2 signaling and contribute to melanomagenesis [89]. Interestingly, a function for SPRY2 in melanoma was recently linked to the ubiquitin ligase SIAH2, which is known to contribute to Sprouty degradation [90]. Inhibition of SIAH2 activity resulted in reduced tumorigenicity. This effect was mediated primarily by the stabilization of SPRY2, which led to an attenuated ERK1/2 signaling [91]. Interestingly, Sprouty proteins are not inevitably considered as negative regulators of RTK signaling. Several studies propose mechanisms for Sprouty proteins that enhance EGFR induced signaling [92], [93]. In this case SPRY2 acts as a decoy for the EGFR-specific ubiquitin ligase CBL and thus seems to prevent CBL-dependent RTK degradation [94].

The SPRED proteins constitute the second branch of the Sprouty family. Although much less is known about the SPREDs compared to the SPRYs, some features have already been described. They are characterized by an N-terminal Enabled/VASP homology 1 domain (EVH1), a central c-Kit (KBD) binding domain and a C-terminal Sprouty domain (SPR), which is common to all Sprouty family members [95].

SPRED1 and SPRED2 seem to execute their function on the level of GTPases like RAS or RHO, either downstream from the GTPases [95] or even upstream [96]. Interestingly, there is recent evidence for SPRED2 to bind to p85 and EGFR. Unexpectedly, these interactions seem to enhance the inhibitory effect of SPRED2 towards ERK1/2 signaling but do not interfere with PI3K signaling [97].

Since the SPRY proteins seem to be implicated in several different malignancies, it is possible that the SPRED proteins can also regulate tumorigenesis. In hepatocellular carcinoma, SPRED2 is implicated in reduced proliferation and even the induction of apoptosis if overexpressed [98]. In Legius syndrome, a disorder that resembles neurofibromatosis-1, mutated SPRED1 was shown to lose the ability to bind neurofibromin, a negative regulator of RAS. This leads to elevates RAS-GTP levels and thus elevated RAS signaling [99]. In case of melanoma, SPRED2 has been identified to be upregulated as a transcriptional target in BRAF and NRAS mutant tumors [67]. However, no functional role has been associated until know. Analysis of Sprouty function in melanoma with regard to resistance mechanisms after genotoxic cisplatin treatment was also part of this work.

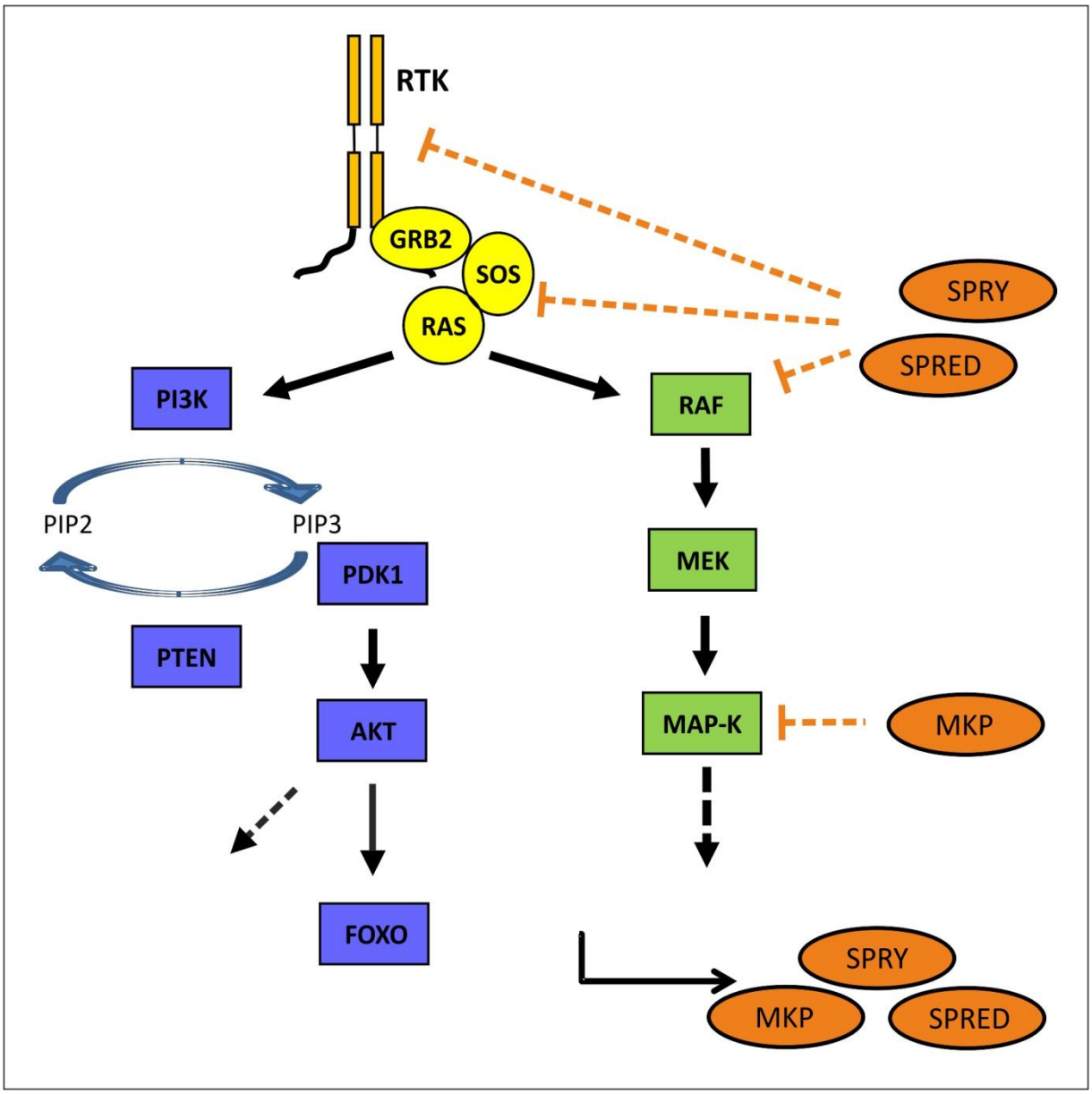

Figure 1: Schematic overview of RTK dependent RAS/PI3K/AKT and RAS/RAF/ERK signaling cascades including negative feedback signaling mechanisms of the latter signal transduction pathway.

3.4 State of the art therapy

Wide local excision is currently the treatment of choice for primary malignant melanomas of stage I or II [100], stages where the disease is restricted to the primary site of tumor development. If the tumor has already progressed to stage III or IV, characterized by lymph node infiltration or metastasis according to the *AJCC Cancer Staging Manual,* an additional treatment is required. Currently there are different forms of therapy. Firstly, since it is known that melanoma is characterized by a high immunogenicity, immunotherapies have been applied as a standard therapy in melanoma using either interleukin-2 (IL2), interferon α (IFNα) [101], or pegylated interferon, which was just approved in 2011 [100]. Additionally, efforts on the development for melanoma specific vaccination seem to develop in a promising direction as seen on clinical trials using MAGE-3 vaccines [102]. The major improvement in the field of immunotherapy, however, was the recent success of the CTLA-4 antibody ipilimumab [103], which prevents CTLA-4 from attenuating the normal T-cell response, thus enhancing the body`s innate anti-tumor response [104]. Ipilimumab was approved by the FDA in 2011.

The second form of melanoma therapy established since decades is chemotherapy. Dacarbazine (DTIC), the first ever approved drug to treat melanoma, and its oral analogue temozolomide, show response rates of 13-20% in metastatic disease [105]. Combination regimen using cisplatin or vinblastine showed no increased benefit [106]. DTIC treatment is considered to be the standard chemotherapy being widely accepted as comparator for novel compounds in clinical trials [107].

The third, although rather rarely applied therapeutic possibility is the usage of irradiation which can be used in combination to chemotherapy or as a palliative treatment for specific subtypes of melanoma [108].

The most recent strategy to treat melanoma is the so-called targeted therapy. Since the discovery of the importance of specific pathways for tumor development and maintenance, a race for the development of specific inhibitors to target these signaling cascades began. Tyrosine kinases are examples for already used druggable targets in other malignancies like chronic myeloid leukemia (CML), where imatinib is successfully employed to inhibit the BCR-ABL fusion protein [109]. In a specific subset driven by the oncogenic receptor tyrosine kinase (RTK) KIT, imatinib is currently also tested in melanoma [110]. Besides this the mitogen activated protein

kinase (MAPK) pathway came in the focus of research efforts. The latter is hyper-activated in 90% of melanoma cases, mostly through mutational activated RAF and RAS isoforms [19], and thus constitutes a pivotal melanoma signaling cascade.

While first generation RAF inhibitors like sorafenib showed no clinical benefit in melanoma [111], major advances have been achieved with second generation BRAF inhibitors like vemurafenib (PLX4032) and dabrafenib (GSK2118436) [112][113]. These inhibitors are characterized by a high specificity towards the most common oncogenic variant of BRAF, $BRAF^{V600E}$. The increased specificity as well as a much higher ability to inhibit ERK1/2 signaling output are the foundations of this success [114]. Overwhelming primary results were achieved using vemurafenib and dabrafenib, with regard to primary response rates, resulting in a strong and fast decrease in tumor burden with a concomitant increase in patient`s life quality and most importantly in progression free survival. However, the treatment was not associated with long-term complete remissions [115]. This is due to emerging primary or secondary inhibitor resistances leading to high relapse rates. Currently the number of described resistance mechanisms is rapidly increasing. They range from upstream activation of MAPK and PI3K signaling through KRAS and NRAS or RTKs like IGF1R, PDGFR, or MET [116] [117–119] to overexpression of COT [120], or expression of an alternative splicing form of $BRAF^{V600E}$ [121]. In this context, reactivation of the MAPK pathway seems to be mediated by either ARAF or CRAF [122]. Interestingly, MEK1 activating mutations were shown to be insufficient in mediating resistance in melanoma [123].

Besides drugs targeting BRAF, highly specific second generation MEK inhibitors are currently succeeding first generation inhibitors like U0126 or PD184352 (CI 1040). Under clinical evaluation are selumetinib (AZD6244) and trametinib (GSK1120212) [107]. In comparison to $BRAF^{V600E}$ inhibition, MEK inhibition could theoretically be used in a much wider panel of melanomas and not only in the fraction that is characterized by the V600E mutation, although these tumors are also prone to MEK inhibitor treatment because of their pathway dependency [124]. However, due to a much lower specificity for tumor cells, MEK inhibitors tend to have more severe side effects. In monotherapy, the MEK inhibitor selumetinib was not able to accomplish the same results as BRAF inhibitors if applied in a cohort without selection for $BRAF^{V600E}$ mutant melanomas. Only in the group of patients with mutated BRAF they showed a slight improvement in response rates compared to temozolomide [125].

Trametinib, however, which was applied in a cohort of patients with $BRAF^{V600E}$ positive melanoma, was able to improve overall survival rates [126]. As expected for specific kinase inhibitors, primary and secondary resistances could be a major drawback for MEK inhibitors as well. However, at this time point there are no data reporting MEK inhibitor resistance development in melanoma. In other malignant settings, amplification of the driving oncogenes (e.g. RAS or RAF), MEK1 mutations or the involvement of the PI3K pathway were described as resistance mediators [127–129]. With ipilimumab and vemurafenib as newly FDA-approved drugs [130], it is the first time within 30 years that there are new treatment possibilities that actually increase the progression free survival time in melanoma [131]. However, upcoming challenges will be the evaluation of co-treatment strategies to increase response rates, progression free and overall survival and to avoid or face emerging drug resistances [132].

This work encompasses the investigation of the benefit of combining the genotoxic agent cisplatin with the MEK inhibitor PD184352 in different melanoma cell lines.

3.5 Aims of the thesis

To be able to propose new or advanced treatment strategies for malignancies like melanoma it is important to understand the basic mechanisms and effects of signal transduction that distinguish malignant cells from their healthy equivalents. The ERK1/2 pathway which is commonly deregulated in melanoma represents a pivotal pathway during melanomagenesis.

One aim of this work was to analyze the function of MKP2 and FOSL1. The coressponding genes are regulated in an ERK1/2 dependent manner and their expression is thus frequently altered in melanoma. Functional consequences of increased or decreased protein abundances of both factors were addressed in this work to get insights into the functions of both proteins.

The possibility to target central signalling pathways like the ERK1/2 pathway with specific inhibitors opened new melanoma treatment possibilities. However, emerging resistances raise the need for further investigation of combination treatments as well as resistance mechanisms. Thus, a second aim of this study was to analyze direct pharmacological inhibition of the ERK1/2 pathway and its role in the regulation of cell survival under genotoxic stress. This part comprises the analysis of a regulatory crosstalk between the ERK1/2 pathway and the AKT pathway in melanoma.

4. Material and Methods

4.1. Material

4.1.1. Cell lines

Cell line	Supplier	Type
A375	ATCC	human melanoma
SK MEL28	ATCC	human melanoma
MeWo	Becker (Würzburg)	human melanoma
Bro	Becker (Würzburg)	human melanoma
IFB	Becker (Würzburg)	human melanoma
A375M	Becker (Würzburg)	human melanoma
Mel Ho	Bosserhoff (Regensburg)	human melanoma
Mel Im	Bosserhoff (Regensburg)	human melanoma
Mel Wei	Bosserhoff (Regensburg)	human melanoma
Dx3	Goding (Oxford)	human melanoma
LT5.1	Goding (Oxford)	human melanoma
WM35	Goding (Oxford)	human melanoma
D10	Hintermann (Basel)	human melanoma
SKMEL 2	NCI/NIH	human melanoma
SKMEL5	NCI/NIH	human melanoma
LOX IMVI	NCI/NIH	human melanoma
RPMI7952	NCI/NIH	human melanoma
M14	NCI/NIH	human melanoma
UACC-257	NCI/NIH	human melanoma
451Lu	Wistar Institute	human melanoma
Hermes3a	Cell Bank Holding	Immortalized human melanocyte
Melan-a	Cell Bank Holding	murine melanocyte
NHEM	ATCC	Primary human melanocyte

4.1.2. Plasmids

Backbone	Insert	source
pBabe	-	
pBabe	MKP2	this work
pBabe	myrAKT3(6xmyc)	Sellers W. (Addgene)
pBabe	GFP	
pCDNA3.1	-	Yoshimura A. [133]
pCDNA3.1	SPRY2WT	
pCDNA3.1	SPRY2DN	
pCDNA3.1	SPRY4WT	
pCDNA3.1	SPRY4DN	
pEF1(A)myc_his	-	Takahashi M. [134]
pEF1(A)myc_his	MKP2	
pRetroSuper	-	
pRetroSuper	MKP2 (human)_shRNA3	this work
pRetroSuper	MKP2 (mouse)_shRNA3	this work

4.1.3. Real-time Primer oligos

Oligo	Sequence
Hu_Actin_for	GGCATCCTGACCCTGAAGTA
Hu_Actin_rev	GGGGTGTTGAAGGTCTCAAA
Hu_BIM_for	TGACACAGACAGGAGCCCAGC
Hu_BIM_rev	CGCCGCAACTCTTGGGCGAT
Hu_BMF_for	CGACTCGGAGGCCGAGACTCTC
Hu_BMF_rev	GCAGTGGGTGAGAGGGAAGAGCT
Hu_CyclinD1_for	GGCCATGCTGAAGGCGGAGG
Hu_CyclinD1_rev	GCTCCAGCGACAGGAAGCGG
Hu_EGF_for	TGACTCTACTCCACCCCCTCACCT
Hu_EGF_rev	AGGTCTCGGTACTGACATCGCTCC
Hu_EGFR_for	GAAGGAGCTGCCCATGAGAA
Hu_EGFR_rev	GACGACCCGTGTCTACTAAAACC
Hu_FOSL1_for	AGCTGCAGAAGCAGAAGGAG
Hu_FOSL1_rev	GGAGTTAGGGAGGGTGTGGT
Hu_IGF1_for	TCCTCTCTGAATCTTGGCTGCTGGA
Hu_IGF1R_for	TGGCCGAGCAGCCCTACCAG
Hu_IGF1R_rev	TCCCGGAAGCCAGGCTCCAT
Hu_IGR1_rev	TTGCTGCTGCTTTTGAGGAGGCC
Hu_MCL1_for	ACTGGGGCAGGATTGTGACTCTCA
Hu_MCL1_rev	TCCTGATGCCACCTTCTAGGTCCTC
Hu_NOXA_for	CTCGCGTCCTGCAGCTGTCC
Hu_NOXA_rev	TGCCGGAAGTTCAGTTTGTCTCCA
Hu_PDGF_for	TGGGAACTGTGCCTGTTGTCTCCA
Hu_PDGF_rev	TGATGCGGCTATCCTCCTGTGCT
Hu_PDGFR_for	GGCCCCACCTGAACGTGGTC
Hu_PDGFR_rev	GAGCCCAACGGGCAGAGCAT
Hu_PUMA_for	CCAGGGCTGCTTCCACGACG
Hu_PUMA_rev	ACACTGCCGAGGGCACCAGG
Hu_RS14_for	CTCAGGTGGCTGAAGGAGAG
Hu_RS14_rev	GCAGCCAACATAGCAGCATA
Hu_SPRED1_for	GGAGACGGCGACTTCTGACAACG
Hu_SPRED1_rev	TCCCTGAGTCGCTCTCCACGG
Hu_SPRED2_for	CCTGGGCCACCTCCACGACT
Hu_SPRED2_rev	GGGTCCGGGTACTTGCCCCT
Hu_SPRED3_for	CGTCATCCACGGGGAACGCC
Hu_SPRED3_rev	GCGCAGCCAGCAGGCTCTT
Hu_SPRY1_for	AGCTGCACCTGGCTCCCACT
Hu_SPRY1_rev	CCCTGCTCCCACGGTACCCA
Hu_SPRY2_for	AGGGGTTGGTGCAAAGCCGC
Hu_SPRY2_rev	TGAGGGCGTCTCTGGGGTCG
Hu_SPRY3_for	GCGCAGCCAGCAGGCTCTT
Hu_SPRY3_rev	TCACCGCAGCATCCATTTTGCCT
Hu_SPRY4_for	AGGGCGGCAAATGCGAGACC
Hu_SPRY4_rev	TGAGGTTGCTCGCCCCCAGA
Hu_TRAIL_for	AGACCTGCGTGCTGATCGTGA
Hu_TRAIL_rev	GACTTGCCAGCAGGGGCTGT

M_Actin_for	GCTACAGCTTCACCACCACA
M_Actin_rev	AAGGAAGGCTGGAAAAGAGC
M_Fosl1_for	TGGAGAAAGGGAGATGCAAG
M_Fosl1_rev	GAGACCGACAAATTGGAGGA
M_MKP2_for	CTGTACCTCCCAGCACCAAT
M_MKP2_rev	GACGGGGATGCACTTGTACT
M_MMP3_for	GTCTTCCGGTCCTGCTGTGGCTGT
M_MMP3_rev	CAGGTTCCAGAGAGTTAGACTTGGTG
M_opn_for	TGCACCCAGATCCTATAGCC
M_opn_rev	CTCCATCGTCATCATCATCG

4.1.4. Antibodies

Primary Antibodies	Manufacturer	Catalog number
Actin ß	Santa Cruz	sc-47778
cleaved caspase3 (Asp175)	Cell Signaling	9664
DUSP4	Protein Tech	10739-1-AP
P-Akt (Ser473)	Cell Signaling	4051
P-Akt (Thr308)	Cell Signaling	9275
P-EGFR (Tyr1173)	Cell Signaling	4407
P-Mapk p42/44	Cell Signaling	9101
P-FoxO1 (Thr 24) / FoxO3a (Thr 32)	Cell Signaling	9464
P-H2AX	Cell Signaling	2577
SPRY1	Sigma-Aldrich	SAB2103834
SPRY2	Sigma-Aldrich	SAB2103944
SPRY4	Sigma-Aldrich	SAB2900414
HA-tag	Cell Signaling	2362
myc-tag	Cell Signaling	2276
P-Elk1	Cell Signaling	9181
P-Mek1/2	Cell Signaling	9121
P-p53	Cell Signaling	9284
P-Tyrosine ("Py20")	BD Transduction Lab.	610000
Tubulin	Sigma	T6074
Erk2 (c-14)	Santa Cruz	sc-154
Fra1 (Fosl1) (N17)	Santa Cruz	sc-183
Fra1 (Fosl1) (C12)	Santa Cruz	sc-28310
Secondary Antibodies	**Manufacturer**	**Catalog number**
Rabbit polyclonal to goat IgG H&L (HRP)	abcam	ab 6741
Goat Anti-mouse IgG+IgM (H+L) (POD)	Thermo Scientific	31444
Goat Anti-rabbit IgG (H+L) (POD)	Bio-Rad	170-6515

4.1.5 siRNAs

siRNA	Manufacturer	Catalog number	Sequence
ON-TARGET plus SMART pool BBC3 (PUMA)	Thermo scientific	L-004380-00-0005	GUAGAUACCGGAAUGAAUU CCUGGAGGGUCCUGUACAA CCGAGAUGGAGCCCAAUUA CGGACGACCUCAACGCACA
siGenome siRNA human SPRY1	Thermo scientific	D-027339-01-0010	GAUAAUCCUUGCUCCUGUU
siGenome siRNA human SPRY2	Thermo scientific	D-005206-02-0010	GCAGGUACAUGUCUUGUCU
siGenome siRNA human SPRY4	Thermo scientific	D-015457-01-0010	GCACGAAUGAGGACGAUGA
siGenome siRNA Non-Targeting	Thermo scientific	D-001210-01-05	n.a.
ON-Target plus Non-Targeting pool	Thermo scientific	D-001810-10-20	n.a.
ON-TARGET plus siRNA FOSL1	Thermo scientific	J-004341-06-0005	GAGCUGCAGUGGAUGGUAC

4.1.6 Inhibitors/ Drugs / compounds

Compound	Manufacturer	Catalog number
AG1478	Calbiochem	658548
LY294002	LC Labs	L7962
PD184352	Axon Medchem	1368
U0126	LC Labs	U-6770
Cholera Toxin	Calbiochem	227035
Endothelin1	Calbiochem	05-23-3800
FuGENE HD Transfection Reagent	Roche	04709713001
ERK Inhibitor	Calbiochem	328006
Recombinant human bFGF	PeproTech	100-18B
G-418 Sulfate	Calbiochem	345812
PLX4032	Axon Medchem	1624
PLX4720	Axon Medchem	1474
Phorbol-12-myristate-13-acetate	Calbiochem	524400
X-tremeGene siRNA Transfection Reagent	Roche	04476093001
puromycin	Calbiochem	540222
hygromycin	Calbiochem	400052
Cycloheximide	Calbiochem	239763
Doxorubicin	Calbiochem	32438
Recombinant human EGF	PeproTech	AF-100-15
Wortmannin	Calbiochem	681675
TKI-258	Selleck Chemicals	S1018
Cisplatin	Calbiochem	232120

4.1.7 Kits

All kits were used according to the manufacturer`s instructions.

Kit	Manufacturer	Catalog number
peqGOLD TriFast 100 ml	PEQLAB	30210
PureYield Plasmid Midiprep System	Promega	A2495
PureYield Plasmid Miniprep System	Promega	A1223
Wizzard SV Gel & PCR Clean-Up System	Promega	A9282
GenElute™ HP Plasmid Miniprep Kit	Sigma-Aldrich	NA0160-1KT
GenElute PCR Clean-Up Kit	Sigma-Aldrich	NA1020-1KT
RevertAid First Strand cDNA Kit	Fermentas	K1622
Cell proliferation ELISA BrdU	Roche	11647229001
SuperSignal West Pico Chemiluminescent Su.	Thermo Scientific	LH146987
Bradford Reagent	Sigma-Aldrich	B6916

4.1.8 Buffer

Buffer	
Laemmli	312,5 mM Tris pH 6.8; 10% SDS, 50% glycerine 0.005% brome-phnole-blue; 25% ß-mercapto-ethanol
PBS	137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4. Adjust to a final pH of 7.4.
TBST	10 mM Tris pH 7.9; 150 mM NaCl; 0.1% Tween
Lysis buffer	20mM HEPES (pH 7.8), 500mM NaCl, 5mM $MgCl_2$, 5mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10mg/ml aprotinin, 10mg/ml leupeptin, 200mM Na_3VO_4,1mM phenylmethanesulphonyl- fluoride and 100mM NaF
ReproFast PCR buffer	100 mM $(NH_4)_2SO_4$; 200mM Tris pH 8.8; 100mM KCl 20mM $MgSO_4$; 1% Triton; 1% BSA
SDS running buffer	250 mM Tris; 192 mM glycine; 0.5% SDS
Transfer buffer	25 mM Tris; 192 mM glycine; 20% methanole

4.1.9 Technical equipment

Photo Image Station 4000MM (Kodak)
Mastercycler ep Realplex (eppendorf)
Mini-PROTEAN Tetra Electrophoresis System (Biorad)
Trans Blot Cell (Biorad)
Cary 50 Spectrophotometer (Varian)
NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies)
Cytomics FC 500 flow cytometer (Beckman Coulter)
Hera Cell 150i Incubator (Thermo Scientific)

4.2 Methods

4.2.1 Maintaining of cell lines

All cell lines were incubated at 37°C under 5% CO_2 in a Hera Cell 150i Incubator (Thermo Scientific). Human melanoma cell lines were maintained in DMEM containing 10% fetal calf serum (FCS) and 1x penicillin streptomycin (PenStrep). Murine melanocytes were maintained in complete medium (DMEM, FCS (10%), 200 nM 12-*0*-tetradecanoyl phorbol acetate (TPA), 200 pM cholera toxin (CT), and 1x PenStrep). Human immortalized melanocytes Hermes 3a were maintained in RPMI 1640 containing 10% FCS, 200 nM TPA, 200 pM CT, 10 ng/ml human stem cell factor, and 10nM endothelin 1. NHEM cells were maintained in HAM`s F10 with 20% FCS, 100 nM TPA, 200 nM CT, 1x PenStrep, 100 µM 3-isobutyl-1-methylxanthine and ITS-Premix (1:1000; BD Bioscience).
All cell lines were passaged before reaching confluence and were regularly tested for mycobacterial contaminations.
For long term storage cells were frozen slowly in freezing medium (DMEM, 20% FCS and 10% dimethylsulfoxide) and stored in liquid nitrogen.

4.2.2 Cloning and construction of vectors

Cloning and construction of vectors, transgenes and shRNA constructs followed standard protocols. Specific shRNA sequences were chosen using siRNA Center (Thermo scientific; http://www.dharmacon.com/designcenter/designcenterpage.aspx).

4.2.3 Establishment of transgenic lines

For the establishment of transgenic cell lines cells were either electroporated, transfected, or infected.
Electroporation was carried out using a Nucleofector II Device (Lonza) with Nucleofector Kit V (Lonza) according to the manufacturer`s instructions.
For Transfection of plasmids, Fugene HD 9 (Roche) was used as transfection reagent according to the supplier`s protocols.

Viral infection of target cells was carried out using either retroviral infection systems (Phoenix cells, http://www.stanford.edu/group/nolan/retroviral_systems/phx.html) according to the inventor`s instructions, or lentiviral systems.
For lentiviral transduction, lentiviral expression vectors (e.g. p201iep) and the helper plasmids pPAX2 and pCMV-VSVG were transfected in 293TD cells. After virus production of 293TD cells, target cells were incubated in virus containing supernatant.
All plasmid transfers were followed by antibiotic selection (puromycin (1-2µg/ml), neomycin (300µg/ml), or hygromycin (350-800µg/ml)) to obtain cultures without non transduced cells.

4.2.4 siRNA transfection

Cells were seeded in 6well dishes and allowed to grow until they reached 60% confluence. SiRNA transfection was performed using XtremeGene reagent (Roche) according to the manufacturers' recommendations. 12 hours after removal of the siRNA and transfection reagent, the cells were reseeded to new dishes. 12-36 hours later additional treatments were conducted if required and subsequent assays were performed as described.

4.2.5 Proliferation assays

To determine the proliferation rate of specific cell lines expressing transgenes or to test the influence of drugs on a cell population`s proliferative capacity, different assays have been performed.
As direct method to determine the changes in numbers of viable cells over time, cell counting was performed. For this, suitable numbers of cells (0.3-2.5 $x10^5$ cells per well on six-well plates) were seeded in triplicate and allowed to grow for two to eight days. Cells were harvested by trypsinization, and were resuspended in 50-2000µl PBS to obtain a countable dilution. A Neubauer hemacytometer was used to count the cells and the actual cell number per well was calculated with respect to the dilution.
As indirect methods to determine proliferation rates either the xCELLigence system (Roche), which utilizes changes in impedance across the well surface, or a Cell

proliferation ELISA BrdU kit (Roche), measuring BrdU incorporation, were used according to the manufacturer`s instructions.
All assays were performed at least in triplicates and significance was determined using Student`s t-test (*: $p<0.05$; **: $p<0.01$; ***:$p<0.001$).

4.2.6 Cell cycle analysis /FACS

Cells were harvested by trypsinization, washed in PBS and fixed in 70% ice-cold ETOH for at least 24 hours. Subsequent rehydration was achieved by two washing steps using PBS. Pellets were suspended in 500 µl 38 mM sodium citrate and treated with 25µl RNaseA (10mg/ml) for 30 min at 37°C. Subsequently, DNA was stained using 8µl propidium iodine (2mg/ml). Flow cytometry was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter). Results were analyzed using CXP software (Beckman Coulter). All assays were performed at least in triplicates and significance was determined using T-test (*: $p<0.05$; **: $p<0.01$; ***:$p<0.001$).

4.2.7 Protein extraction, SDS-PAGE and Western blot

Cells were collected from dishes by either trypsinization or with help of a silicone rubber and were lysed in lysis buffer. Protein concentration was determined photometrically using Bradford Reagent (Sigma-Aldrich) and a Cary 50 Spectrophotometer (Varian). 50µg of protein (diluted in lysis buffer), denatured in Laemmli buffer, were dissolved on 8%-20% polyacrylamide gels and transferred to nitrocellulose membranes (Hartenstein). Membranes were blocked in 5%BSA in TBST and incubated overnight at 4°C in blocking solution containing diluted primary antibodies at the manufacturers recommended dilutions. Several washing steps (TBST) were performed and the membranes were incubated with HRP-coupled secondary antibodies (diluted in 5%BSA in TBST) for one hour. After another washing period, detection was carried out using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and a Photo Image Station 4000MM (Kodak).

4.2.8 RNA extraction, cDNA synthesis, and Real-time PCR

Cells were collected from dishes and pellets were dissolved in peqGOLD TriFast Reagent. RNA was isolated DNA free and precipitated according to the supplier`s manual. Residual DNA contaminations were removed by 30 min DNase I, RNase free (Fermentas) digestion. RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). 1-4µg of cDNA was reversely transcribed from the isolated RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer´s instructions.
25ng of cDNA were used for real-time PCR. Reactions were performed in triplets and mRNA abundance was normalized to a housekeeping gene (*RS14* or *Actin*). PCRs were conducted and analyzed using a Mastercycler ep Realplex (Eppendorf).

A standard reaction was prepared as follows:

14,25µl H_2O
2.5µl 10xBuffer (ReproFast)
0.3µl Polymerase
0.7µl dNTPs (10mM)
0.75µl SYBR-GREEN 1:2000
0.75µl 5’ primer (10pmol/µl)
0.75µl 3’ primer (10pmol/µl)
5µl cDNA (5ng/µl)

Standard cycling program was performed as follows:

Step		**Temp.**	**Time**
1		95°C	pause
2		95°C	5’
3	40 repeats	95°C	15’’
4		60°C	15’’
5		72°C	15’’
6		95°C	5’
7		60°C	15’’
8		60°-95° gradient	20’
9		95°C	15’’

All assays were performed at least in triplicates unless stated otherwise and significance was determined using Student’s t-test (*: $p<0.05$; **: $p<0.01$; ***:$p<0.001$)

5 Results

5.1 Functional validation of Xmrk targets in the HERmrk model and in human melanoma

A microarray based approach to identify transcriptional targets of Xmrk signaling in the murine melanocyte based HERmrk system led to the identification of *Fosl1* and *Mkp2* as strongly upregulated genes [55]. Aim of this part of the study was to identify the regulation as well as putative functional features of FOSL1 and MKP2 not only in the murine melanocyte model but also in human melanoma cell systems.

5.1.1 FOSL1

Since Xmrk and thus HERmrk is positioned upstream of several distinct signaling cascades besides the ERK1/2 cascade (reviewed in [135]), the immediate dependence of *Fosl1* transcription on ERK1/2 signaling activity was addressed in HERmrk^{me} cells, which are characterized by a moderate HERmrk expression level and high proliferation rates after EGF stimulation. In fig. 2A the relative amount of *Fosl1* transcript is depicted. HERmrk signaling was triggered by addition of hEGF. After two and eight hours of stimulation, *Fosl1* transcript levels peaked with approximately eight fold induction compared to unstimulated controls. Additional administration of the MEK inhibitor U0126 abolished any HERmrk induced expression. To investigate FOSL1 protein levels, HERmrk^{me} cells were starved and again stimulated with hEGF for 24h (fig. 2B). This induced phosphorylation of ERK1/2 as well as upregulation of FOSL1. Both effects could be prevented in the presence of U0126. Starved HERmrk^{me} cells and the parental cell line melan-a displayed comparable levels of P-ERK1/2 as well as FOSL1. This demonstrates the robustness of the EGF inducible HERmrk-construct.

To examine FOSL1 expression in human melanoma cells, the amount of FOSL1 protein was addressed in a panel of melanoma cell lines with different oncogenic background (BRAF as well as NRAS mutated cell lines). In comparison to primary neonatal human epidermal melanocytes (NHEM), half of the analyzed lines (A375M DX3 LT5.1, SKMEL3, SKMEL28, IFB and A375) showed a strong expression of FOSL1, while the other lines showed comparable or even reduced (Mel Ho, Mel Im,

Mel Wei) levels (fig. 2C). For the following studies a FOSL1 high expressing cell line (A375) and a low expressing cell line (Mel Ho) were chosen.

In both tested human melanoma cell lines incubation with U0126 for 24h abolished P-ERK1/2 as well as FOSL1 levels, demonstrating ERK1/2 pathway dependence of FOSL1 protein expression in the human system (fig 2D). As previously shown for NHEM cells, the non-transformed but immortalized melanocyte cell line Hermes3a, displayed a virtually undetectable level of endogenous FOSL1.

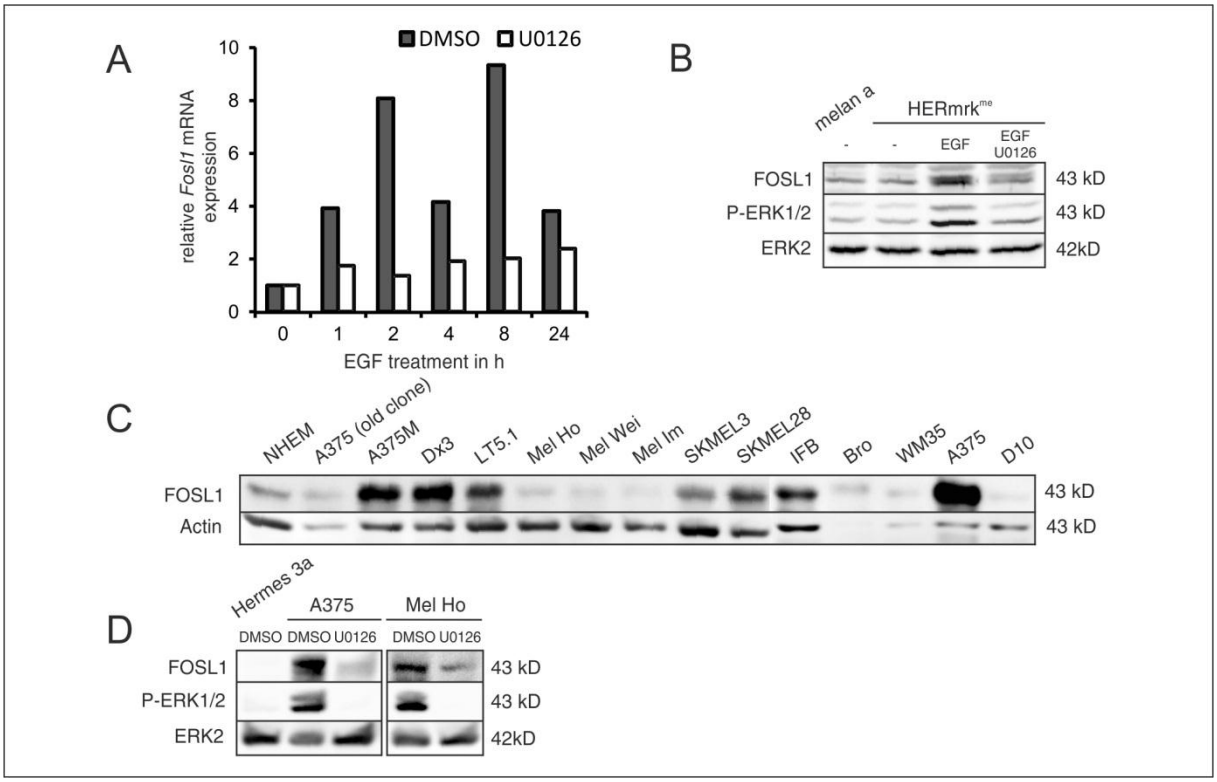

Figure 2: FOSL1 expression and ERK1/2 signaling dependency in murine and human melanoma systems **A.** Relative *Fosl1* mRNA expression levels after EGF stimulation in HERmrk^{me} cells at the indicated time-points (displayed data represents a single experiment; for additional data see [55]). **B.** Western blot analysis of Fosl1 protein levels in HERmrk^{me} cells or parental cells. Cells were treated with EGF and U0126 for 24h. P-ERK1/2 (T202, Y204) and ERK2 levels served as control for inhibition and equal loading, respectively. **C.** Western blot analysis of FOSL1 protein levels in a panel of regularly passaged human melanoma cell lines. Actin served as loading control. **D.** Western blot analysis of FOSL1 in Hermes3a, A375 or Mel Ho cells. Cells were treated with DMSO or U0126 as indicated. P-ERK1/2 and ERK2 levels served as control for inhibition and equal loading, respectively. (**B.** and **D.** modified from [55])

To analyze whether FOSL1 has a functional effect in human melanoma, an siRNA mediated knockdown of *FOSL1* was performed. In comparison to scrambled (scr) siRNA, transfection of *FOSL1*-specific siRNA reduced the levels of the target mRNA by 86% and 71% in A375 and Mel Ho cells, respectively (fig. 3A). In addition to that, the strong downregulation of Fosl1 expression could also be observed on the level of protein as depicted in fig. 3B. Functional effects of the knockdown could be observed

on migration [55] as well as on proliferation (fig. 3C). To determine relative proliferation rates, incorporation of BrdU during six hours of incubation was measured. In both tested cell lines the amount of incorporated BrdU was reduced by 21% and 23% in A375 and Mel Ho cells. However, 48 h after siRNA transfection, no obvious change in the cell cycle distribution in *FOSL1* knockdown cells in comparison to control siRNA transfected cells could be observed (fig. 3D).

Taking together, increased levels of FOSL1 in melanoma are regulated by the ERK1/2 pathway and have enhancing effects on proliferation and migration.

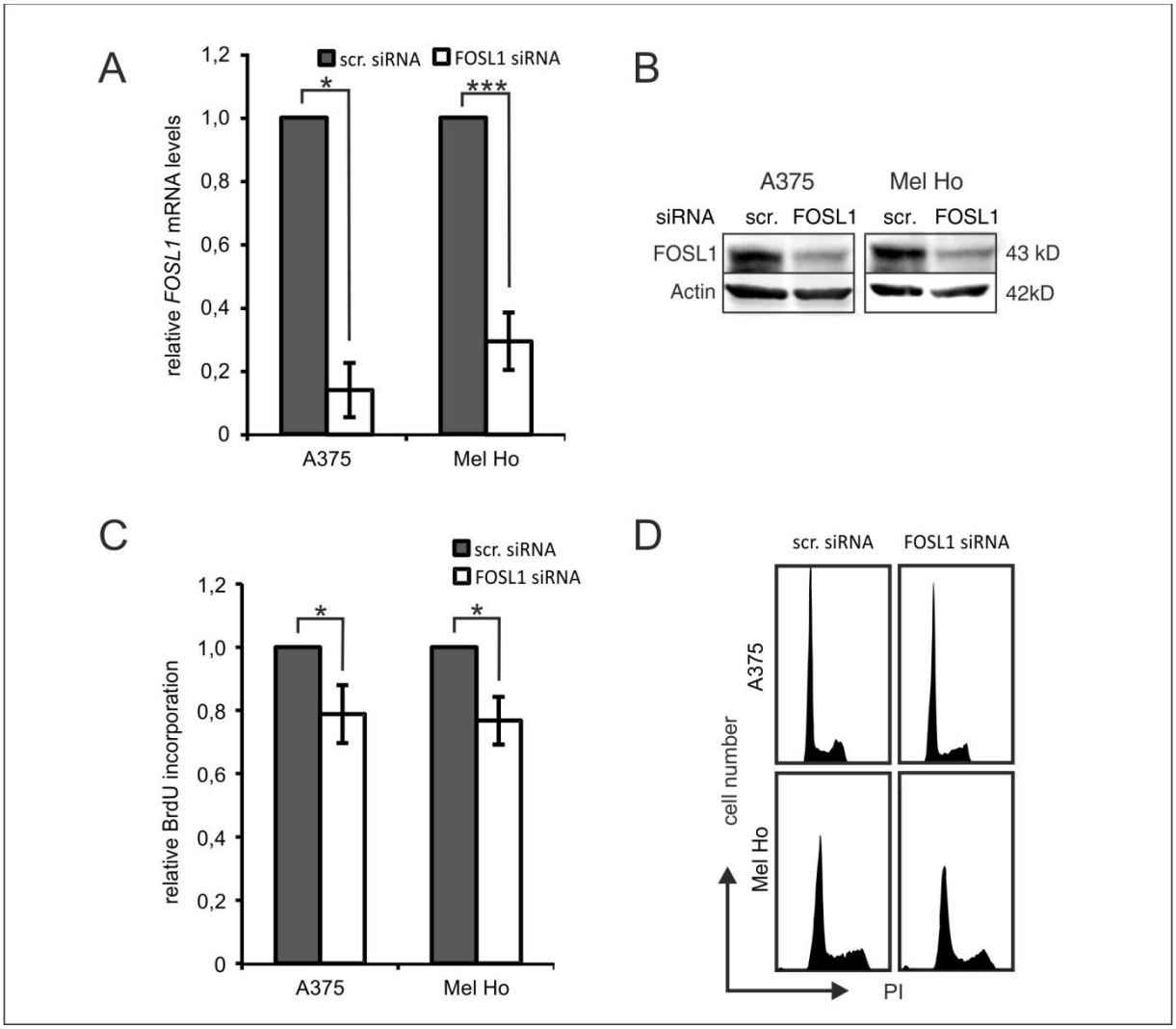

Figure 3: siRNA mediated *FOSL1* knockdown in A375 and Mel Ho cells. **A.** Relative *FOSL1* mRNA expression levels 48h after the cells were transfected with *FOSL1*-specific or scrambled (scr) siRNA. **B.** Western blot analysis of FOSL1 protein levels 48h after siRNA transfection. Actin levels served as loading control **C.** Relative BrdU incorporation of *FOSL1*-specific or scr siRNA transfected cells. Cells were incubated with BrdU for six hours at 48h after siRNA transfection. **D.** Flow cytometric analysis of propidium iodine (PI) stained cells 48h after siRNA transfection. (**A.-C.** modified from [55])

5.1.2 MKP2

Another ERK1/2 associated target gene identified in the HERmrk microarray was *Mkp2* [55]. *Mkp2* knockdown experiments in HERmrk cells were conducted in order to identify functional features of MKP2 in the murine melanocyte model.

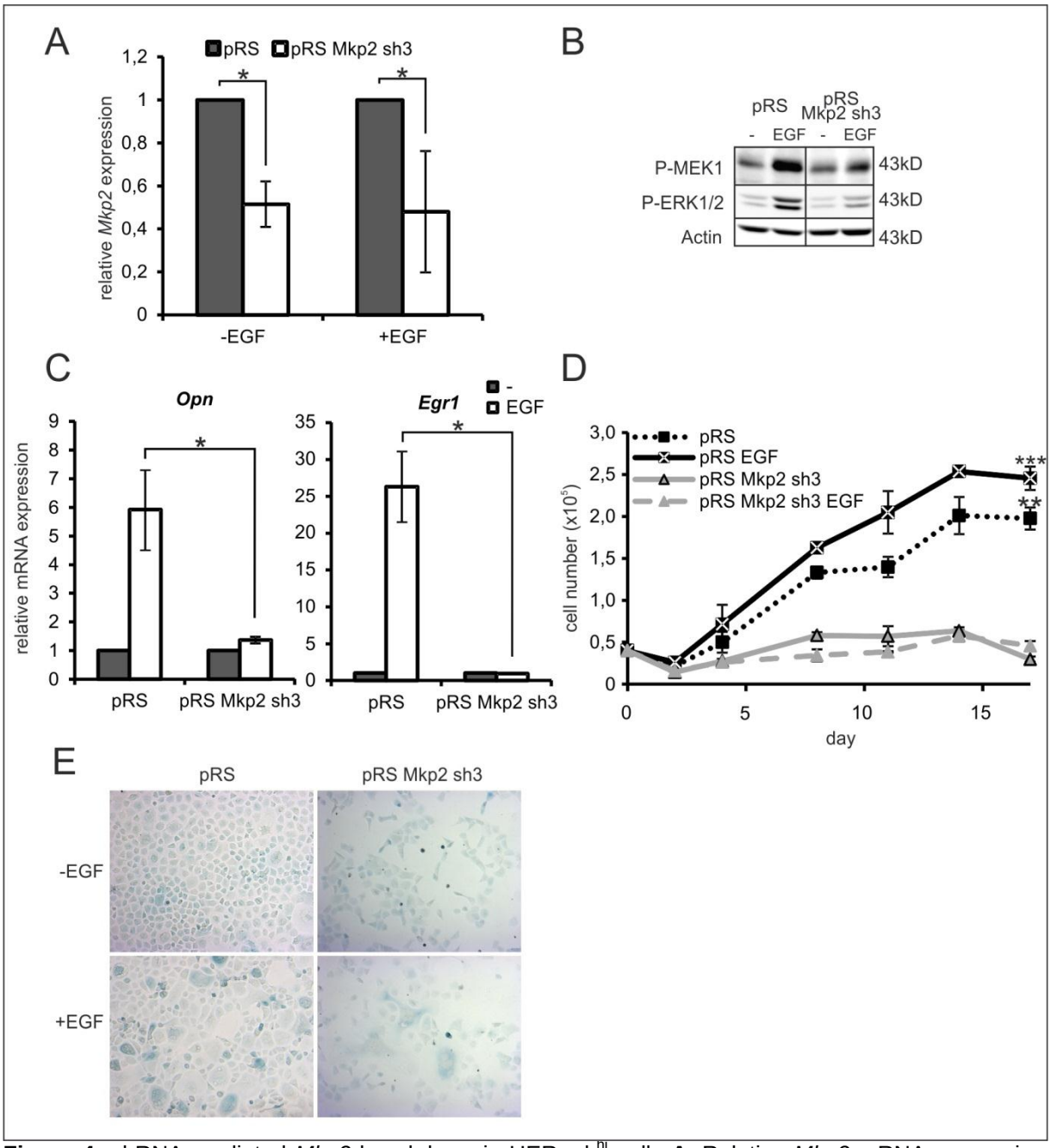

Figure 4: shRNA mediated *Mkp2* knockdown in HERmrk^{hi} cells **A.** Relative *Mkp2* mRNA expression levels in in HERmrk^{hi} cells stably transfected with *Mkp2* specific shRNA (pRS Mkp2 sh3) expressing or control (pRS) vector. Cells were either starved or treated with EGF for 8h **B.** Western blot analysis of phosphorylated MEK1/2 and ERK1/2 (T202, Y204) in *Mkp2* knockdown and control cells. Actin levels served as loading control. **C.** Relative *Opn* (left) and *Egr1* (right) mRNA expression levels in control or *Mkp2* knockdown cells after 8h of EGF treatment. **D.** Determination of cell numbers in *Mkp2* knockdown and control cells. Cells were either starved or stimulated with EGF and counted manually at the indicated time-points. **E.** Senescence associated ß-galactosidase (SA-ß-Gal) staining of Mkp2 knockdown and control HERmrk^{hi} cells after 14 days of EGF stimulation.

The HERmrk^{hi} clone is characterized by the expression of very high HERmrk levels. As a consequence, it displays a strongly reduced proliferation rate compared to the above mentioned HERmrk^{me} cells, but instead it shows senescence after activation of HERmrk signaling [54]. Since regulation of ERK1/2 signaling strength is important in the induction of senescence [54] [136], and MKP2 activity is known to have an influence on ERK signaling, HERmrk^{hi} cells were chosen for the knockdown experiments to be able to analyze the role of MKP2 in the induction of senescence. After cloning of several specific MKP2 shRNA constructs, the *Mkp2* knockdown was performed using MKP2 sh3, constitutively overexpressed by the pRetroSuper (pRS) vector. As control, empty vector infected cells were used. In fig. 4A the knockdown efficiency was verified under starved as well as EGF stimulated conditions. In both cases the knockdown reduced the target mRNA levels to approximately 50% relative to control cells. Firstly, effects of reduced *Mkp2* levels on the phosphorylation levels of the MKP2 target ERK1/2, which were expected to increase after *Mkp2* knockdown, were analyzed (fig.4B). EGF stimulation strongly induced MEK1 and ERK1/2 phosphorylation in control cells. Surprisingly, in the *Mkp2* knockdown, induction of the ERK1/2 pathway was less pronounced. Secondly, the known ERK1/2 targets *osteopontin* (*Opn*) and *early growth response1* (*Egr1*) [55] were analyzed with regard to their expression levels after HERmrk signaling (fig. 4C). Both genes were strongly induced in control cells (6-fold and 27-fold for *Opn* and *Egr1* respectively). However, in cells expressing the *Mkp2*-specific shRNA, induction was completely abolished. The reduced activation of the MEK/ERK1/2 pathway by *Mkp2* knockdown was unexpected and implicates a more complex regulation mechanism of MKP2 than previously anticipated. Thirdly, the proliferation rate of starved and stimulated control and *Mkp2* knockdown cells was analyzed (fig. 4D). While in control cells the proliferation rates could be slightly induced by EGF treatment, this effect could not be observed in cells bearing the *Mkp2* knockdown. Furthermore, proliferation under starved conditions was strongly reduced in the knockdown compared to the control situation. Finally, the appearance of senescent calls was monitored using senescence associated ß-galactosidase staining (SA-ß-Gal). Fig. 4E displays SA-ß-Gal stained, starved, and EGF stimulated control as well as *Mkp2* knockdown cells after 14 days of cultivation. While control cells strongly showed the induction of senescence in a high proportion of cells, the knockdown cells display a much lower overall cell density but no obvious change in the proportion of senescent cells.

To analyze the Mkp2 sh3 mediated effects in non-senescent cells, effects of the knockdown was additionally analyzed in HERmrk^{me} cells.

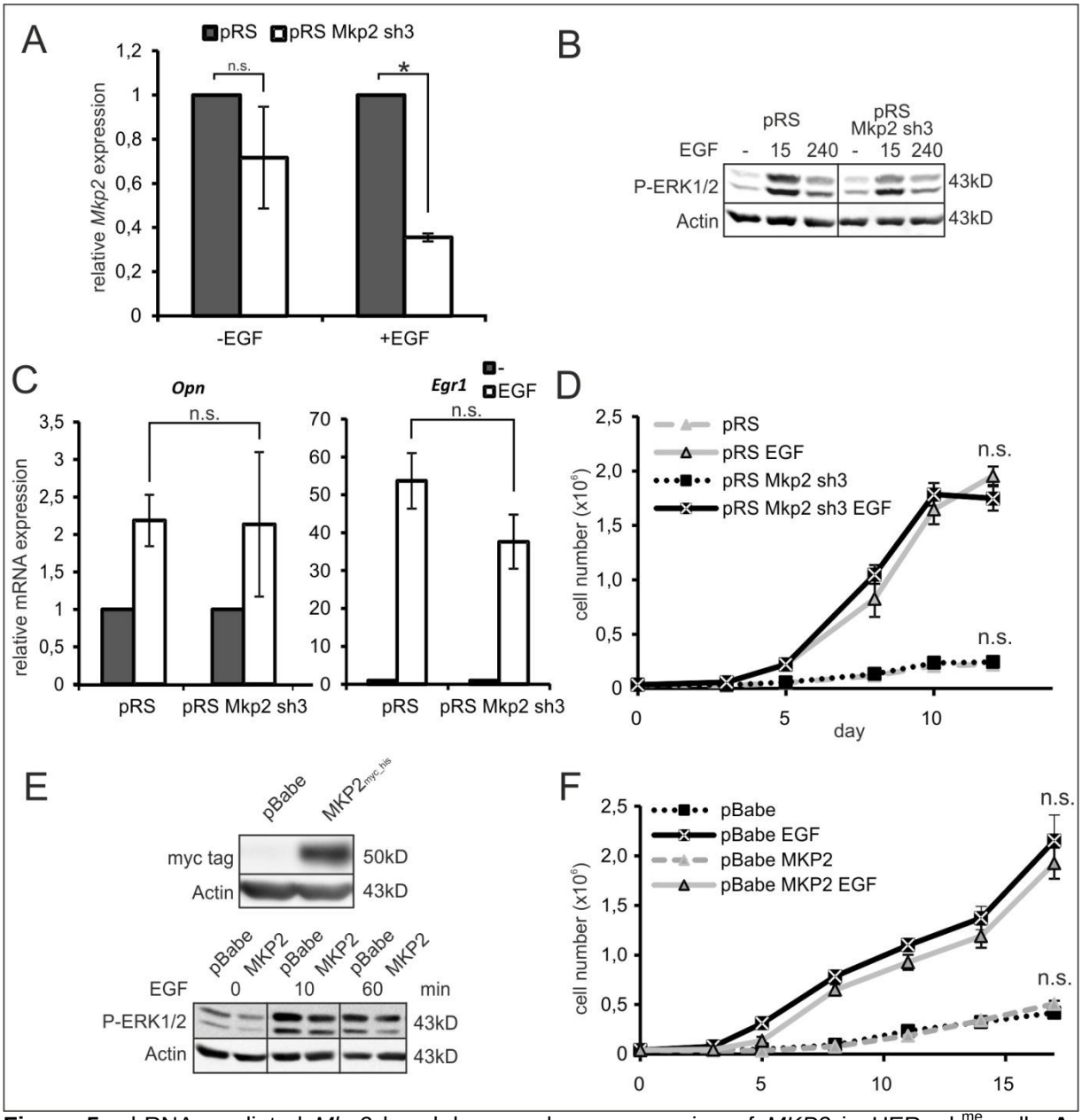

Figure 5: shRNA mediated *Mkp2* knockdown and overexpression of *MKP2* in HERmrk^{me} cells **A.** Relative *Mkp2* mRNA expression levels in HERmrk^{me} cells stably transfected with *Mkp2* specific shRNA (pRS Mkp2 sh3) expressing or control (pRS) vector. Cells were either starved or treated with EGF for 8h. **B.** Western blot analysis of phosphorylated ERK1/2 (T202, Y204) in *Mkp2* knockdown and control cells. Actin levels served as loading control .**C.** Relative *Opn* (left) and *Egr1* (right) mRNA expression levels in control or *Mkp2* knockdown cells after 8h of EGF treatment. **D.** Determination of cell numbers in *Mkp2* knockdown and control cells. Cells were either starved or stimulated with EGF and counted manually at the indicated time-points. **E.** Western blot of tagged human MKP2 (anti myc tag) in HERmrk^{me} cells infected with MKP2 overexpression vector (MKP2$_{myc_his}$) or control vector (pBabe) (upper blot). Levels of phosphorylated ERK1/2 were determined (lower blot). Actin levels were determined as loading control. **F.** Determination of cell numbers in *MKP2* overexpressing and control cells. Cells were either starved or stimulated with EGF and counted manually at the indicated time-points.

In starved as well as EGF stimulated cells the shRNA mediated knockdown could be confirmed (fig 5A). Expression levels of *Mkp2* were reduced to 75% and 40% respectively. Similar to the situation observed in HERmrk^{hi} cells, EGF stimulation strongly induced phosphorylation of ERK1/2 in control as well knockdown cells after 15 min of stimulation (fig. 5B). However, no obvious change of P-ERK1/2 levels could be observed in the knockdown. Analysis of the expression levels of the ERK1/2 dependent HERmrk target genes *Opn* and *Egr1* revealed a robust induction after EGF stimulation in control as well as knockdown cells (fig. 5C). No remarkable differences in expression strength in knockdown or control cells could be observed. Analysis of proliferation rates showed identical growth in starved control and knockdown cells and an identical proliferation rates in cells with induced HERmrk signaling (fig. 5D). To address whether the overexpression of *MKP2* would allow insights into the function of MKP2 in HERmrk induced melanomagenesis, a MKP2 overexpression vector (pBabe MKP2) was cloned and transfected. Cells expressing the empty vector (pBabe) were used as control. The overexpression could be verified by detection of the myc-tagged, overexpressed MKP2 as shown in fig. 5E (upper blot). In contrast to the knockdown situation, a change in P-ERK1/2 levels became evident in the MKP2 overexpressing cells (fig. 5E, lower blot). As expected from an ERK1/2 negative feedback component, reduced phosphorylation levels in the overexpressing cells could be observed after 10 and 60 minutes of EGF stimulation as well as on the basal phosphorylation levels as seen in the starved, non EGF-stimulated situation. Analysis of proliferation levels, however, revealed no differences between control and knockdown cells (fig. 5F). In both cell lines induction of proliferation after EGF treatment could be clearly observed. Taking together, MKP2 seems to be of high importance only in those murine melanocytes that express particularly high levels of HERmrk.

To identify a putative function of MKP2 on the proliferation of human melanoma cell lines, stable knockdown as well as overexpression approaches were used. The knockdown was achieved using a human *MKP2*-specific shRNA vector (pRS MKP2 sh3). The efficiency of the knockdown in the cell lines A375 and Mel Ho are depicted in fig. 6A. *MKP2* mRNA levels were reduced to 48% and 61%, respectively. Analysis of proliferation rates showed no difference between knockdown and vector control in the cell line Mel Ho (fig. 6B).

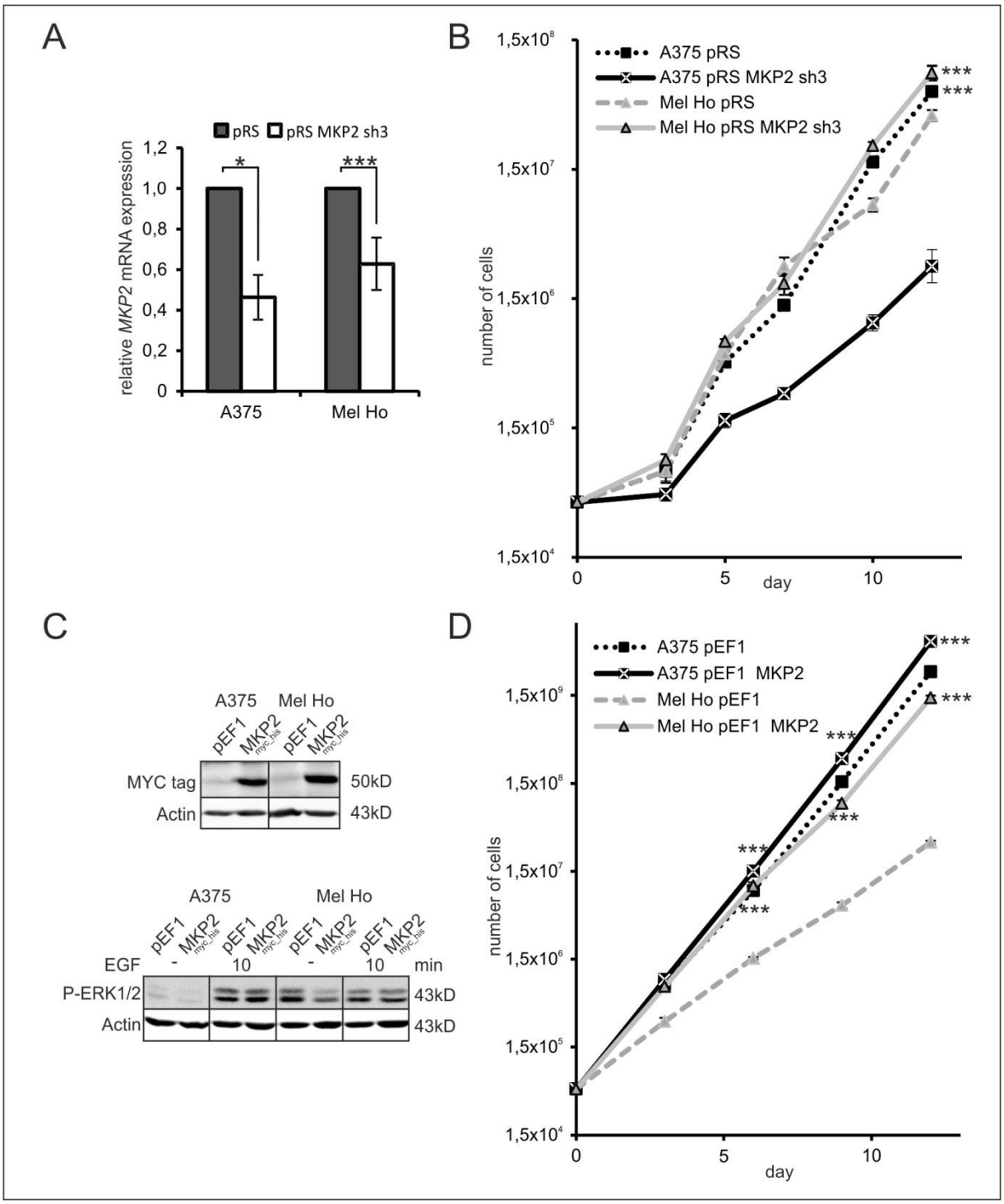

Figure 6: shRNA mediated *MKP2* knockdown and overexpression of MKP2 in A375 and Mel Ho cells **A.** Relative *MKP2* mRNA expression levels in A375 and Mel Ho cells stably transfected with a MKP2 specific shRNA (pRS MKP2 sh3) expressing or control (pRS) vector. **B.** Determination of cell numbers in *MKP2* knockdown and control A375 and Mel Ho cells. Cells were maintained under standard conditions and counted manually at the indicated time-points prior to diluted reseeding (note logarithmic scaling). **C.** Western blot detection of tagged *MKP2* (anti myc tag) in A375 and Mel Ho cells infected with *MKP2* overexpression vector (pEF1 $MKP2_{myc\_his}$) or control vector (pEF1) (upper blot). Levels of phosphorylated Erk1/2 (T202, Y204) were determined (lower blot). Actin levels were determined as loading controls. **D.** Determination of cell numbers in *MKP2* overexpressing and control A375 and Mel Ho cells. Cells were maintained under standard conditions and counted manually at the indicated time-points prior to diluted reseeding (note logarithmic scaling).

In contrast, A375 cells transfected with the *MKP2*-specific shRNA vector showed a strongly reduced proliferation rate compared to the control.

To further address the observed changes in proliferation rates, the MKP2 overexpression vector (pEF.1 MKP2) was transfected in A375 and Mel Ho cells. Fig. 6C (upper blot) illustrates the overexpression efficiency. The myc-tagged MKP2 construct could be detected using myc specific antibodies. Furthermore, in Mel Ho cells, a decrease of the basal phosphorylation level of ERK1/2 under starving conditions could be observed (fig. 6C). This effect got abolished after ten minutes of EGF stimulation. While the *MKP2* overexpression in A375 cells led to a slight increase in cell growth, a much stronger effect of growth promotion could be observed in Mel Ho cells (fig. 6D).

Taken together, the functional role of MKP2 seems to be highly diverse in different cellular settings as evident by the various described effects of the knockdown and overexpression in the different cell systems. A higher expression of MKP2 had no effect on HERmrk^{me} melanocytes, but was generally more beneficial for HERmrk^{hi} melanocytes and human melanoma cells. However, the effect of MKP2 on P-ERK1/2 levels was different in HERmrk^{hi} and melanoma cells.

5.2 MEK inhibition prevents cisplatin induced apoptosis in a AKT and PUMA dependent manner

To directly investigate the effect of ERK1/2 signaling on melanoma cells, the pathway was engaged directly using a pharmacological MEK inhibitor. Targeting the MAPK signaling axis is a well pursued strategy in the treatment of melanoma. However, the likely emerge of resistances imply the necessity to develop drug combination regimen suitable to effectively target tumor growth and maintenance.

The following experiments were conducted to analyze the *in vitro* efficacy of MEK inhibition alone and a combined application of a MEK inhibitor and the genotoxic chemotherapeutic cisplatin as a representative pro-apoptotic agent in order to get insights into the role of MAPK signaling in a chemotherapeutic treatment situation.

5.2.1 MEK inhibition in melanoma cells

Firstly, the effect of ERK1/2 pathway inhibition on a panel of melanoma cell lines was addressed. The phosphorylation levels of ERK1/2, the single described MEK1/2 target, were effectively reduced throughout the treatment period of four days in all tested cell lines. Fig. 7A displays P-ERK1/2 levels two and four days after start of the experiment. Thus, the applied concentration of 2 µM PD184352 was sufficient to inhibit the RAS/RAF/ERK MAPK signaling cascade. Notably, LOX IMVI and A375 cells show a strongly reduced, but detectable faint band of phosphorylated ERK1/2 in presence of PD184352 after 96h of treatment.

Fig. 7B and C display representative images of cell lines treated with DMSO or the MEK inhibitor PD184352 (2µM) for four days. Four (LOX IMVI, A375, RPMI7951, and MeWo) of the eleven tested cell lines showed a slightly reduced number of living cells at the experiment`s endpoint (fig.7B), constituting a group of MEK inhibitor insensitive cells, while the remaining seven cell lines (SK MEL5, M19 MEL, SK MEL28, UACC-62, 451Lu, Mel Ho, and SKMEL2) were characterized by strongly reduced numbers of viable cells in the PD184352 treated fraction (fig. 7C). These cells belong to the PD184352 susceptible cohort.

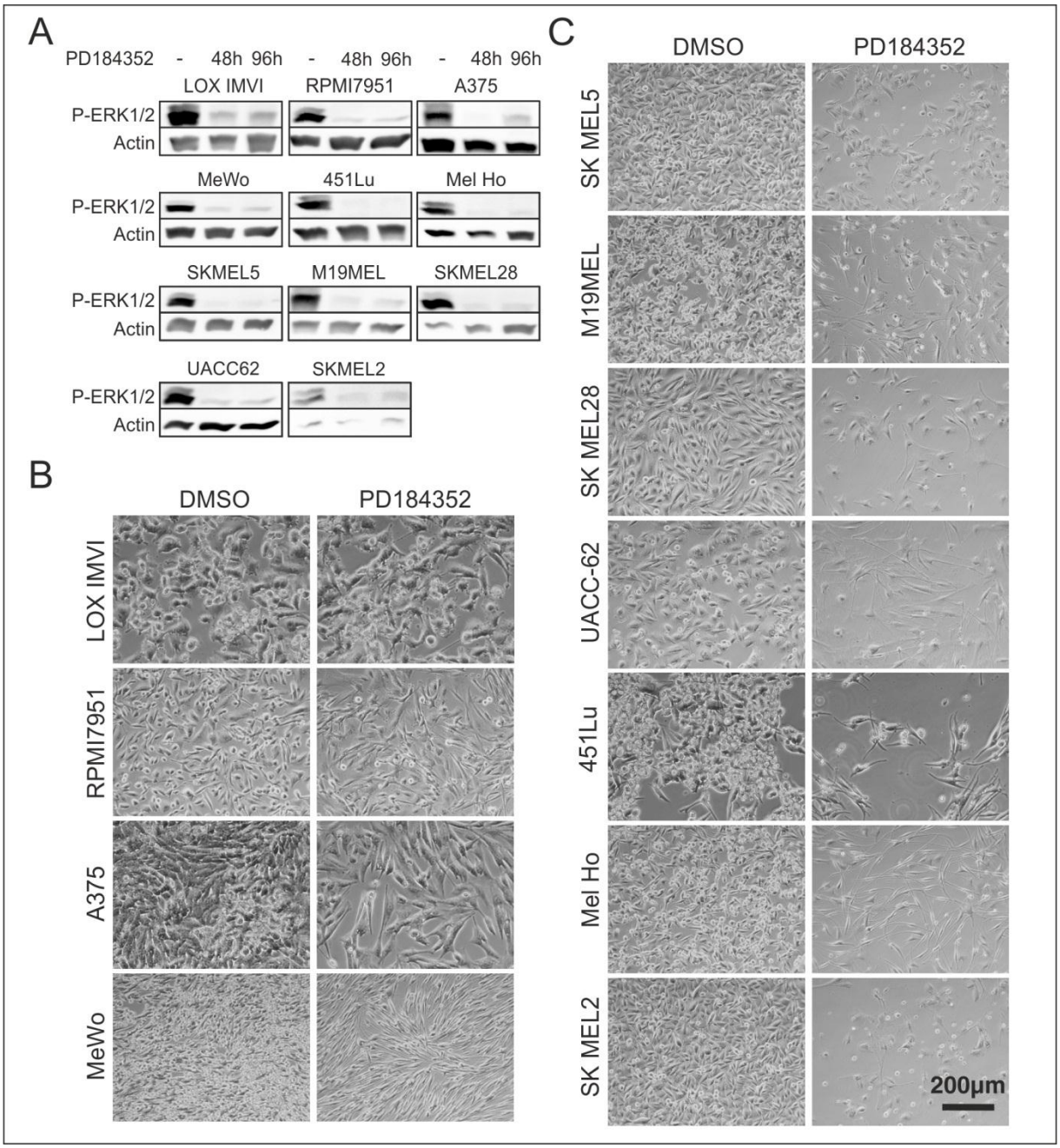

Figure 7: PD184352 mediated MEK inhibition in a panel of eleven human melanoma cell lines. Cells were treated with 2µM PD184352 or DMSO for 96h. **A.** Western blot determination of P-ERK1/2 (T202, Y204) levels in the tested melanoma cell line panel after 48h or 96h of 2µM PD184352 treatment **B.** Representative images of cell lines that primarily show cell cycle arrest after 96h PD184352 treatment (LOX IMVI, A375, RPMI7951, MeWo) **C.** Representative images of cell lines that primarily show induction of apoptosis after 96h PD184352 treatment (SK MEL5, M19 MEL, SK MEL28, UACC-62, 451Lu, Mel Ho, and SKMEL2)

Quantification of the change in cell number during the four days of treatment (fig. 8A) showed that cells from the insensitive group kept proliferating in presence of PD184352, though at a lower rate compared to the DMSO control. Compared to that, members of the PD184352 susceptible group were characterized by a reduction in cell number, hinting at the induction of apoptosis.

To analyze the consequences of MEK inhibition further, the effects of different concentrations of PD184352 on a subset of six melanoma cell lines was determined (fig. 8B). All six tested cell lines showed a reduced number of living cells at all tested inhibitor concentrations (0,5-4µM) in comparison to untreated cells. 451Lu and Mel Ho cells, belonging to the susceptible group, were characterized by a remarkably lower amount of viable cells even at the lowest tested PD184352 concentration.

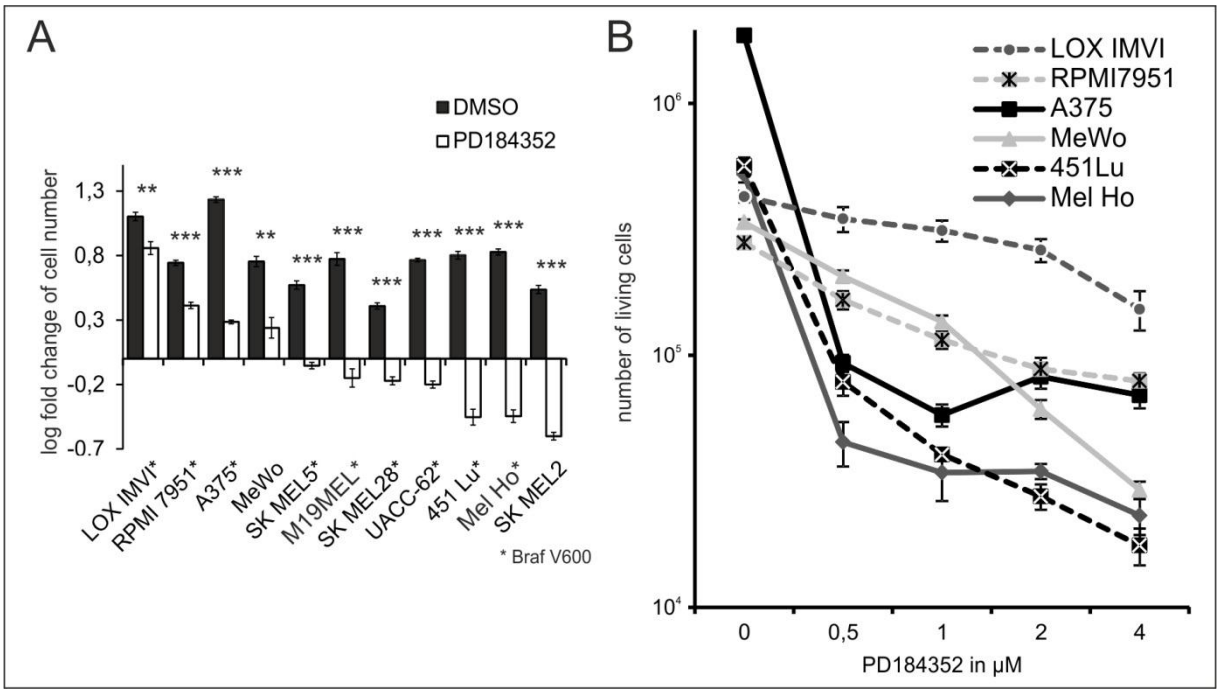

Figure 8: Inhibition efficiency of PD184352 **A.** Fold change of cell number after 2µM PD184352 or DMSO treatment relative to the number of cells seeded prior to treatment start (note: log fold change is displayed). **B.** Concentration dependent effect of PD184352 on six melanoma cell lines (LOX IMVI, RPMI7951, A375, MeWo, 451Lu and Mel Ho). Number of living cells was manually determined after 96h of PD184352 treatment of the indicated concentrations (note logarithmic scaling).

To be able to distinguish between reduced proliferation rate and the induction of apoptosis, cell cycle profiles after four days of MEK inhibitor treatment were compiled and accumulation of cells in subG1 was monitored (fig. 9A). Six cell lines (fig. 9A, left) were chosen for these experiments. Among them were the four members of the MEK inhibitor insensitive cohort (LOX IMVI, A375, RPMI7951 and MeWo) as well as two cell lines representing the high-susceptibility group (451Lu, Mel Ho). While the former four cell lines show only a modest proportion of cells in subG1 (5%, 12%, 11% and 6% respectively) the highly susceptible lines show a much higher number of cells in the subG1 fraction (62% and 77% respectively, fig. 9B, upper panel).

In addition to the increase in subG1, an accumulation of viable cells in G0/G1 was observed in all cell lines except A375 (fig. 9B, lower panel). The increase of the

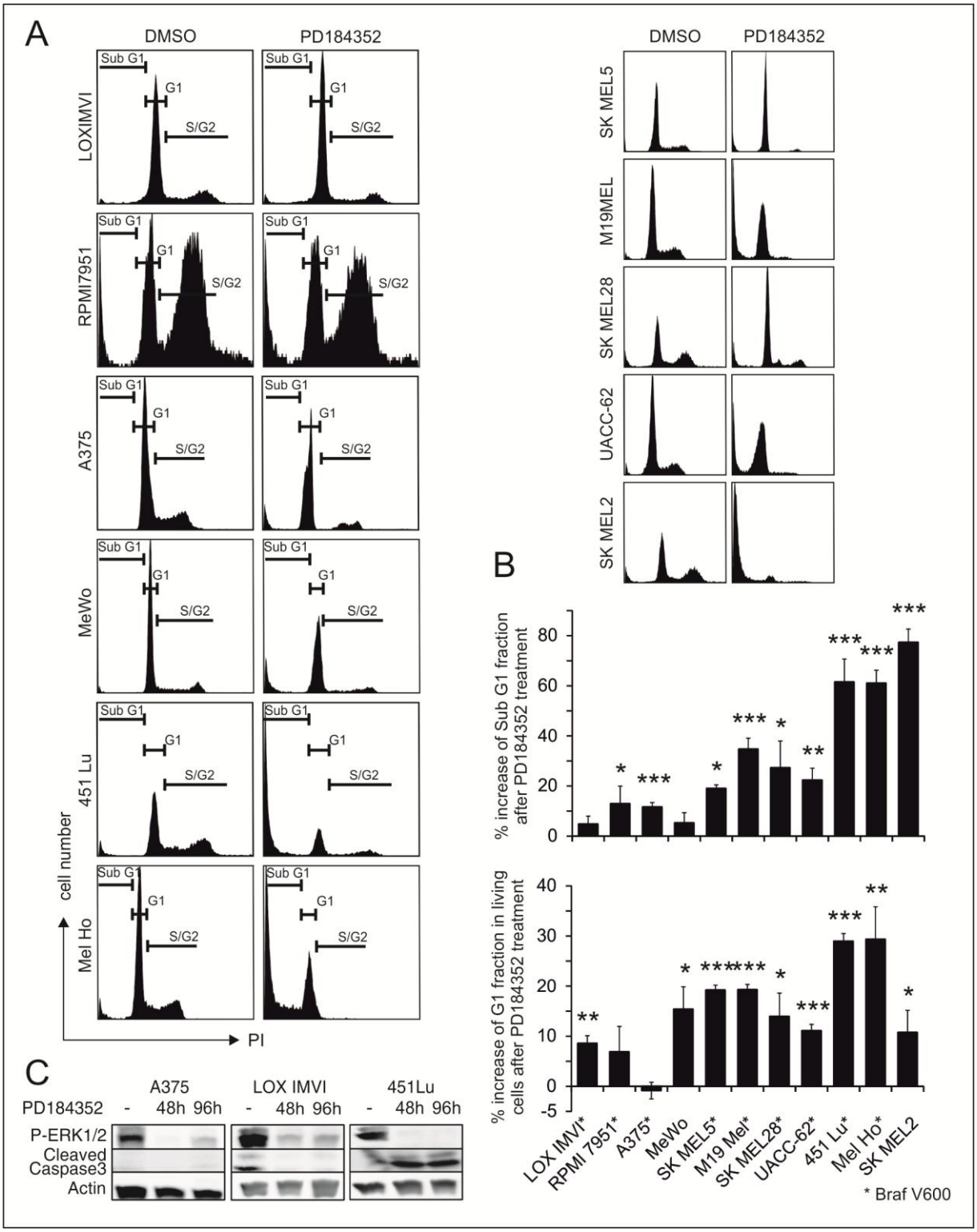

Figure 9: Analysis of melanoma cells treated with 2µM PD184352 or DMSO for 96h **A.** Cell cycle profiles. Cells and supernatant containing detached cells were collected and the DNA was stained using propidium iodine (PI). Cell-cycle profiles were determined using a flow-cytometer. **B.** % increase of cells in the apoptotic (Sub G1) fraction **(upper panel)**. % increase of non-SubG1 cells in G1 **(lower panel)**. Quantified from A. **C.** Western blot analysis of A375, LOX IMVI and 451Lu cells treated with PD184352 for the indicated periods. Lysates were blotted and probed with ERK1/2 (T202, Y204) and cleaved caspase3 antibody. Actin served as loading control.

G1 fraction in the non-apoptotic cell population ranged from 8% (RPMI7951) to 39% (Mel Ho). To test whether the observed cell death was caused by apoptosis, the levels of the apoptotic effector cleaved caspase3 were determined in LOX IMVI, A375 and 451Lu cells by Western blot (fig. 9C). While in LOX IMVI and A375 cells PD184352 treatment did not result in presence of cleaved caspase3, a strong induction of this apoptosis effector could be observed in 451Lu cells.
As a conclusion, the sole inhibition of the MAPK pathway only caused substantial cell death in some melanoma cell lines, thus arguing against the application of MEK inhibitors in monotherapy.

5.2.2 Cisplatin and MEK inhibitor co-treatment in melanoma

It has previously been observed that RAF inhibition by sorafenib enhanced the effects of dacarbazine, a DNA alkylating drug [182]. Thus, I addressed whether MEK inhibition is able to sensitize melanoma cells to the pro-apoptotic agent cisplatin, which efficiently inhibits DNA replication. For this, melanoma cell lines were cultivated in the presence of cisplatin alone or cisplatin and PD184352. The different treatment regimen were carried out for four days in the six cell lines chosen during the previous experiments described in section 5.2.1 (LOX IMVI, A375, RPMI7951, MeWo, 451Lu and Mel Ho). A strong cytostatic effect of cisplatin mono-application could be observed in all tested cell lines as evident by the low density of living cells remaining after four days of cisplatin treatment (fig 10A). In addition to that, high proportions of apoptotic cells could be detected throughout, as shown by the cell cycle profiles (fig. 10B, for DMSO control see fig. 9A). In two of the tested cell lines (451Lu and Mel Ho) an additive effect of cisplatin and PD184352 was observed. This became evident by the reduced number of living cells in the MEK inhibitor/cisplatin co-treatment situation compared to the cisplatin mono-application (fig. 10A, C) and by the high increase of cells in the subG1 fraction (55% and 31% respectively) under the latter condition (fig. 10B, D). Unexpectedly, three of the six tested cell lines (LOX IMVI, A375, and RPMI7951) showed an increased cell number in the co-treatment situation compared to the cisplatin mono-application (fig. 10A, C). This effect was accompanied by a reduction of cells in the subG1 fraction in the PD184352/cisplatin co-treated cells (8%, 18%, and 30% and respectively; fig. 10B, D).

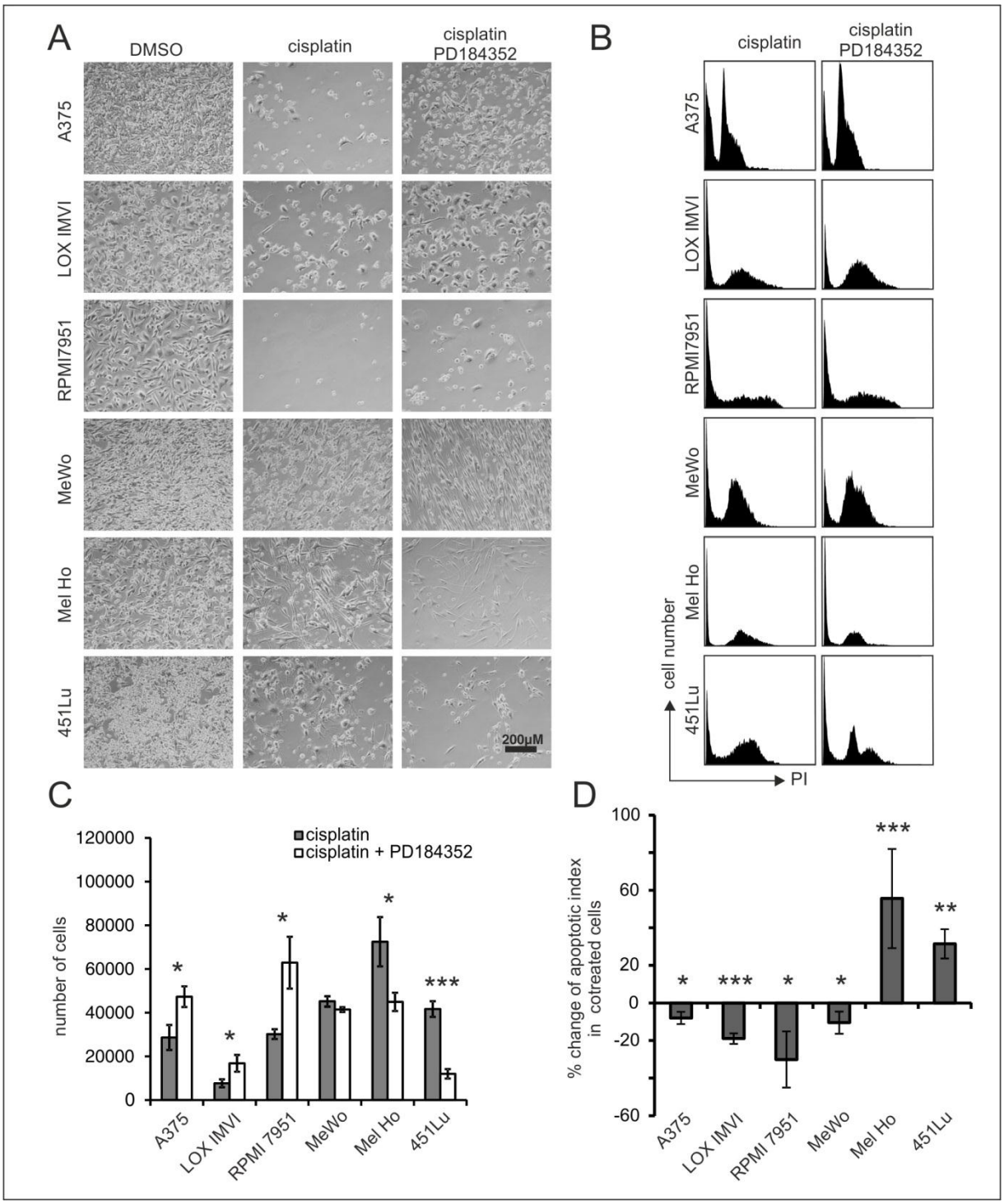

Figure 10: Cisplatin (10µM) and PD184352 (2µM) co-treatment for 96h in human melanoma cell lines (A375, LOX IMVI, RPMI7951, MeWo, Mel Ho and 451Lu) **A.** Representative images of cells treated with DMSO, cisplatin or both. **B.** Cell cycle profiles of indicated cell lines in response to cisplatin or cisplatin and PD184352. Cells and supernatant (containing detached cells) were collected and the DNA was stained using propidium iodine (PI). The cell cycle profiles were determined using a flow-cytometer. **C.** Number of living cells in cisplatin and either PD184352 or DMSO treated cells **D.** Change of the apoptotic index in cells co-treated with cisplatin/DMSO or cisplatin/PD184352. The apoptotic index describes the % change of cells in subG1 in the co-treated cells referred to cisplatin mono-treatment.

To analyze this further, the activation state of the major apoptosis-regulating melanoma pathways was investigated by Western blot (fig. 11). In all tested cell lines,

the levels of phosphorylated ERK1/2 were effectively reduced by PD184352 regardless of the presence of cisplatin. To check for cisplatin-induced apoptosis, the levels of cleaved caspase3 were determined. In accordance with the results obtained by flow cytometry, those cell lines that showed a reduced level of cells in SubG1 (LOX IMVI, A375 and RPMI7951), were also displaying strongly reduced levels of cleaved caspase3 in the co-treatment situation compared to cisplatin mono-application. This effect was also observed in MeWo cells. Contrastingly, cleaved caspase3 levels were weakly reduced in Mel Ho cells and increased in 451Lu cells after additional administration of PD184352. In 451Lu cells the MEK inhibitor alone was able to induce apoptosis (fig. 9C, 11).

Interestingly, in LOX IMVI, A375 and RPMI7951 cells, the MEK inhibitor-induced desensitization towards cisplatin was accompanied by concomitant phosphorylation and thus activation of AKT (fig. 11). This was observed in presence as well as absence of cisplatin. Furthermore, phosphorylation of the AKT target proteins FOXO1/3a was increased, too. AKT-dependent FOXO1/3a phosphorylation at position Thr24/32 leads to cytoplasmic sequestration and thus inactivation of the transcription factor [137]. In MeWo, Mel Ho and 451Lu cells, AKT and FOXO1/3a phosphorylation largely paralleled ERK1/2 phosphorylation levels and were thus reduced after MEK inhibition.

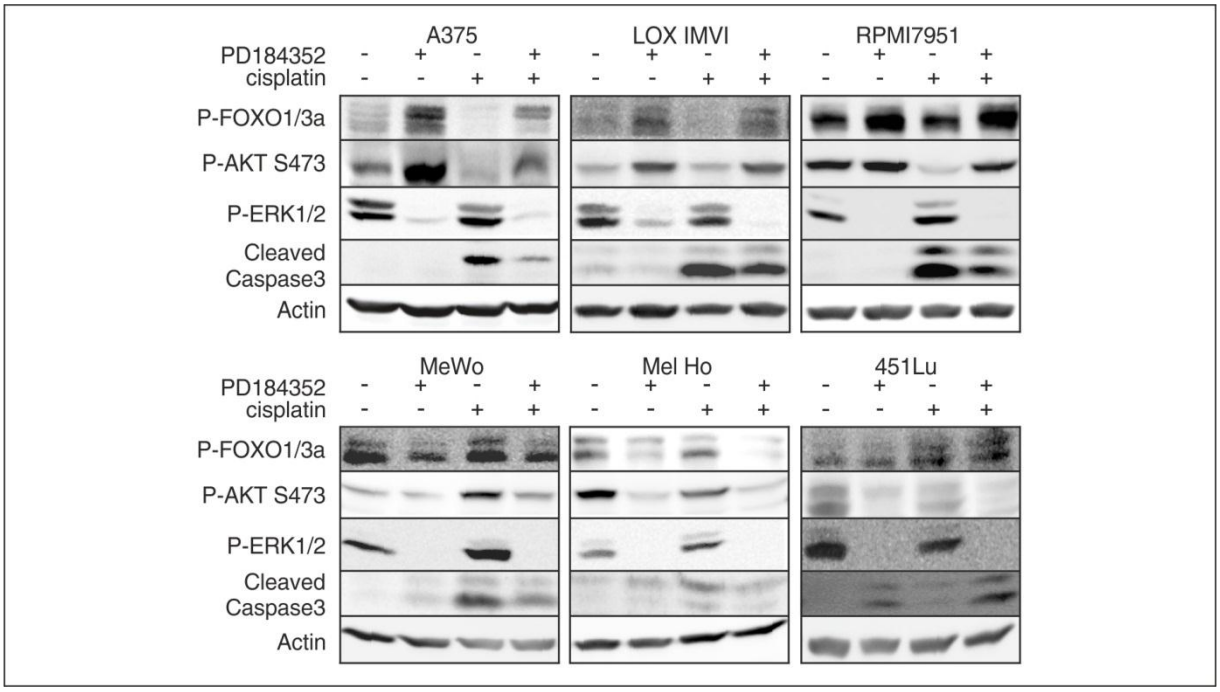

Figure 11: Western blot analysis of human melanoma cell lines co-treated with cisplatin (10µM) and PD184352 (2µM) for 48h. Protein lysates were blotted and probed for P-FOXO1/3a (T24/32), P-AKT (S473), P-ERK1/2 (T202,Y204), cleaved caspase3 and the loading control β-actin.

These data raise the question whether the MEK inhibitor induced AKT pathway activation is responsible for the protection of the respective melanoma cells from cisplatin-induced cell death.

5.2.3 AKT activation in response to MEK inhibition

The observed crosstalk between the ERK and AKT pathways leading to activation of AKT after MEK inhibition was detected 48 hours MEK inhibitor treatment. To analyze if this effect is a primary feedback mechanism occurring downstream of receptor tyrosine kinases, the pathway was investigated in the murine melanocyte cell line HERmrk^{me} as well the human melanoma cell line A375 (fig. 12A). Both cell lines were starved for 24h and stimulated with EGF (HERmrk^{me}) or FCS (A375) in presence or absence of the MEK inhibitor U0126. In both cell lines the levels of P-ERK1/2 were reduced effectively by U0126 treatment. Upon stimulation of the cells, MEK inhibition was accompanied by AKT phosphorylation at all tested time points.

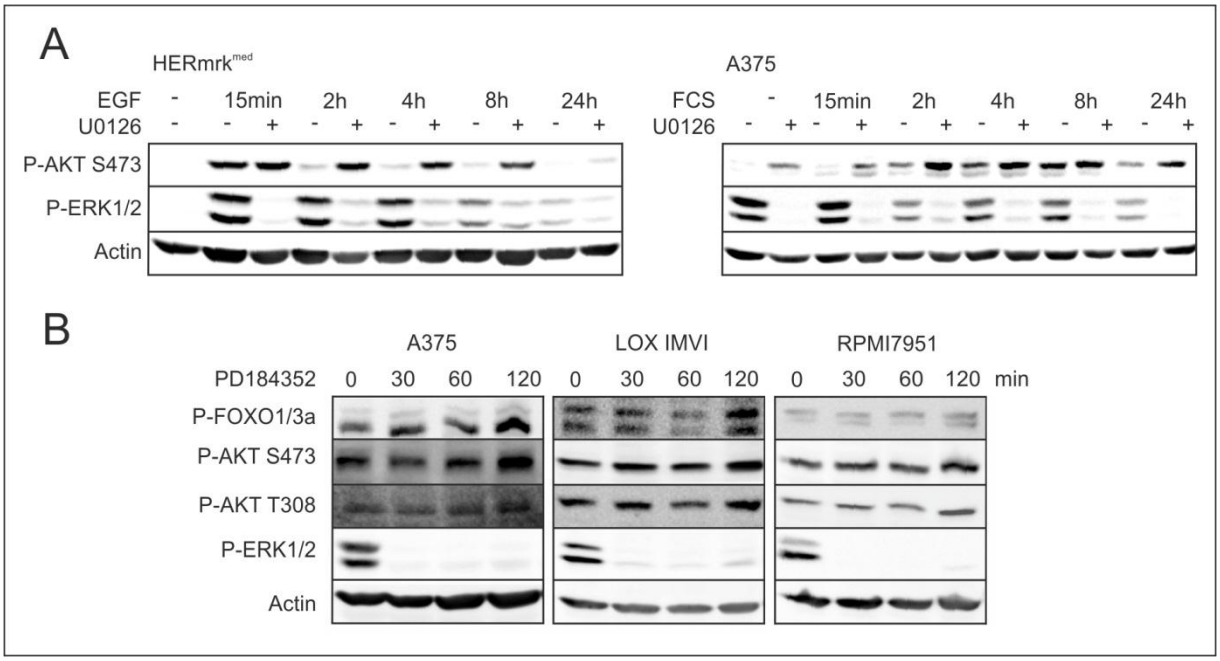

Figure 12: Western blot analysis of MEK inhibition dependent upregulation of P-AKT levels. **A.** HERmrk^{me} cells (**left**) and A375 cells (**right**) were starved for 24h and treated with EGF (100ng/ml) or FCS (10%) in absence or presence of U0126 for the indicated time points. Levels of P-ERK1/2 (T202, Y204) and P-AKT (S473) were determined. β-actin was used as loading control. **B.** A375, LOX IMVI and RPMI7951 cells were treated with PD184352 for the indicated time points. Levels of P-FOXO1/3a (T24/32), P-AKT (S473), P-AKT (T308), and P-ERK1/2 (T202, Y204) were determined. β-actin served as a loading control.

To get a better insight into the mechanism and kinetics of MEK inhibitor induced P-AKT upregulation, the three melanoma cell lines A375, LOX IMVI and RPMI7951 were treated with PD184352 for short time periods between 30 and 120 minutes under normal (non-starved) conditions. Fig. 12B demonstrates that in all three cell lines MEK inhibition robustly abolished ERK1/2 phosphorylation at all time points. In addition to that, increased phosphorylation levels of AKT at serine 473 and threonine 308 and of FOXO1/3a was visible after 120 minutes in all three cell lines.

Since phosphorylation of AKT at threonine 308 has been described to be a possible upstream event leading to serine 473 phosphorylation and because, in addition threonine 308 phosphorylation is directly dependent on PDK1 and thus PI3K activity, the actual PI3K dependency of the observed crosstalk was addressed.

Firstly, long term treatment of different melanoma cell lines with LY294002, an inhibitor of PI3K, were performed in the presence of cisplatin (fig. 13A) to address whether the application of a PI3K inhibitor would be suitable to avert the apoptosis preventing effect of MEK inhibition.

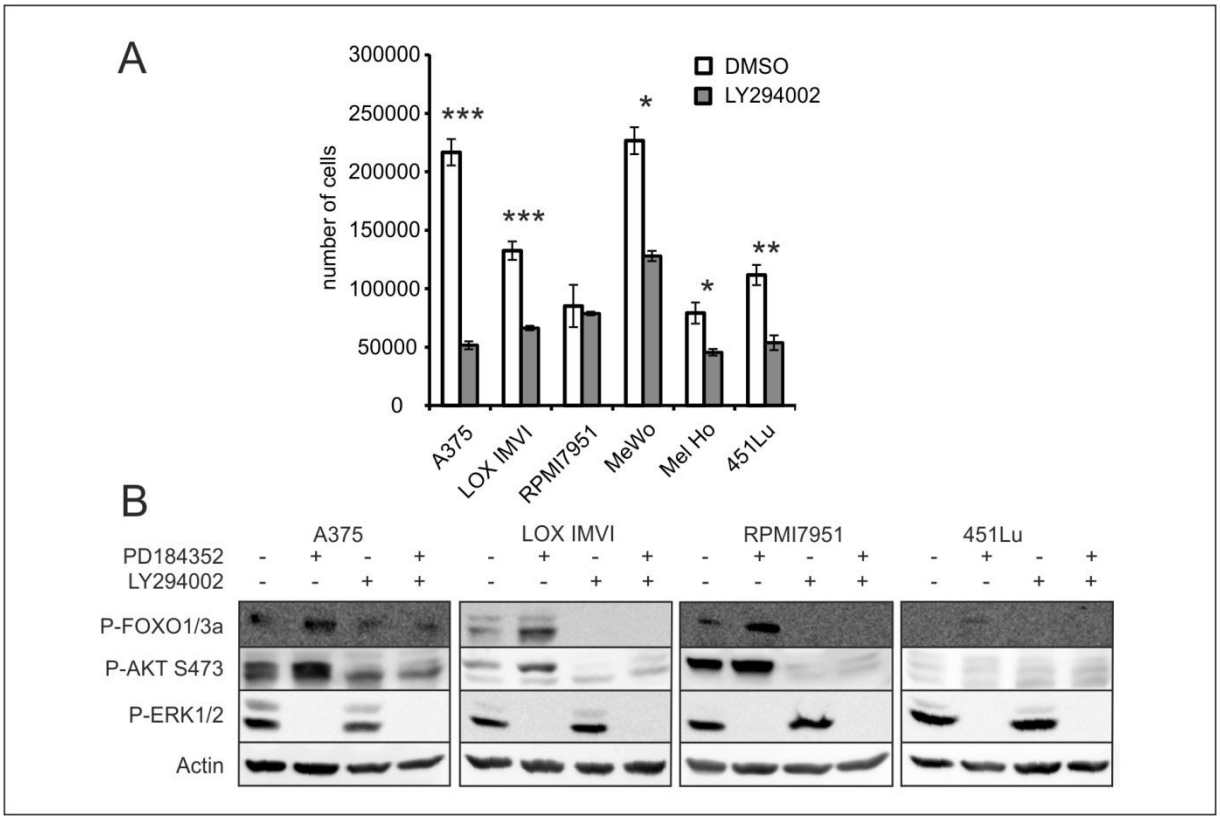

Figure 13: Role of PI3K on MEK inhibition dependent upregulation of P-AKT levels**. A.** Number of living cells in cisplatin (7.5µM) and DMSO or LY294002 (20µM) co-treated cells. Cells were treated for 96h and cell number was determined manually. **B.** Western blot analysis of A375, LOX IMVI, RPMI7951, and 451Lu cells treated with PD184352 (2µM) and LY294002 (20µM) for 24h. Levels of P-FOXO1/3a (T24/32), P-AKT (S473), and P-ERK1/2 (T202, Y204) were determined. β-actin served as a loading control.

However, it turned out that LY294002 alone had a strong effect on cell numbers in all tested cell lines and enhanced cell death induced by cisplatin. Therefore, this approach was not suitable to address the PI3K/AKT contribution to the apoptosis preventing effect of MEK inhibition. Nevertheless, LY294002 could be used to address the kinetics of AKT phosphorylation in absence or presence of PD184352 in short time experiments (fig. 13B). In A375, LOX IMVI and RPMI7951 cells treatment with PD184352 for 24h induced serine 473 phosphorylation of AKT. Additional administration of LY294002 prevented this effect completely.

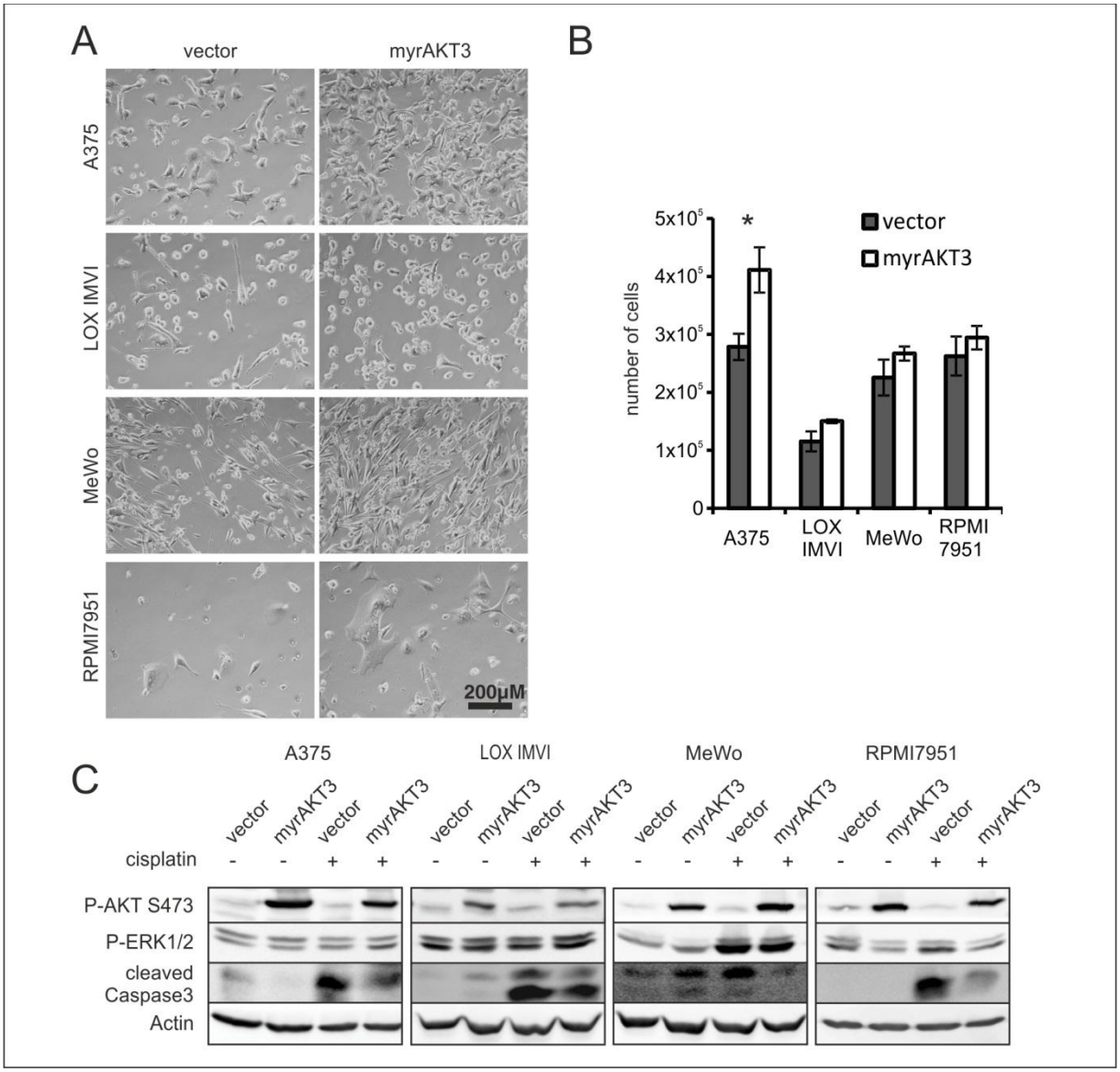

Figure 14: Constitutive active myristoylated AKT3 mimics MEK inhibition in A375, LOX IMVI, MeWo, and RPMI7951 melanoma cell lines **A.** Representative images of cells stably infected with a constitutive active version of AKT3 (myrAKT3) or control vector (vector) treated with cisplatin (10µM) for 24h. **B.** Number of living cells in cisplatin (10µM) treated myrAKT3 and control cells. Cells were treated for 96h and cell number was determined manually. **C.** Western blot analysis of myrAKT3 and control cells treated with cisplatin for 24h. Levels of P-AKT (S473), and P-ERK1/2 (T202, Y204) and cleaved caspase3 were determined. β-actin served as a loading control.

Secondly, to directly address the capability of activated AKT in preventing genotoxically induced apoptosis, cells were transfected with a constitutively active form of AKT3 (myrAKT3). Cells stably expressing myrAKT3 as well as control cells were challenged with cisplatin. In fig. 14A representative images of transgenic A375, LOX IMVI, MeWo and RPMI7951 cells are displayed. After 24h, in A375 and MeWo cells the fraction of surviving cells was clearly increased in cells carrying the constitutively active variant of AKT3. In LOX IMVI and RPMI7951 cells this effect was also visible, albeit much weaker. A four-day incubation with cisplatin resulted in slightly increased numbers in living cells in the myrAKT3 fraction, reaching significance only for LOX IMVI cells (fig. 14B). Furthermore, the levels of cleaved caspase 3 were determined in cells treated with cisplatin for 24h (fig. 14C). In all four tested cell lines, cleaved caspase3 was induced by cisplatin. However, in cells expressing myrAKT3, this effect was strongly diminished. Additionally, the proper functionality of myristoylated AKT3 could also be observed on the basis of the high levels of serine 473 phosphorylation of AKT, since this site can be activated by AKT itself. In A375 and LOX IMVI cells no change in ERK1/2 phosphorylation by either myrAKT3 or cisplatin was detected. In MeWo cells, however, cisplatin treatment increased P-ERK1/2 levels, while in RPMI7951 cells high AKT activity slightly reduced P-ERK1/2 levels.

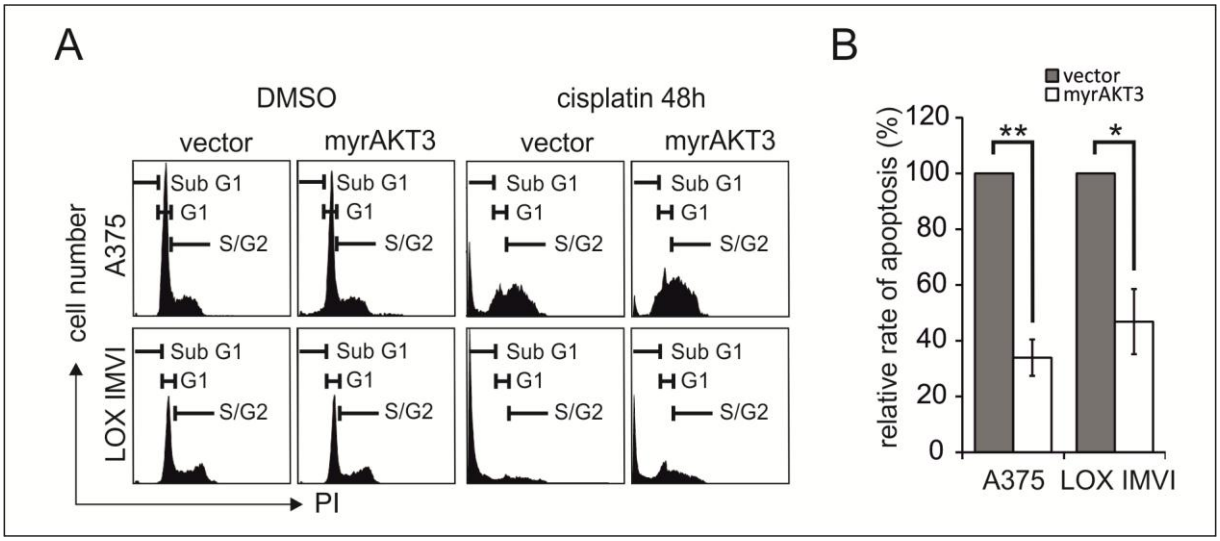

Figure 15: Constitutively active myristoylated AKT3 protects from cisplatin induced apoptosis in A375 and LOX IMVI cell lines **A.** Cell cycle analysis of myrAKT3 and vector control cells. Cells with supernatant containing detached cells were collected after 48h of cisplatin (10µM) treatment. Containing DNA was stained using PI. Cell-cycle profiles were determined using a flow-cytometer. **B.** Reduction of apoptotic fraction in myrAKT3 cells compared to vector control cells. Quantification of A.

Lastly, the increased survivability of myrAKT3 expressing cells in comparison to control cells was demonstrated by analysis of cell-cycle profiles. MyrAKT3 expressing A375 and LOX IMVI cells were treated with cisplatin for 48h and subjected to flow cytometric DNA content analysis (fig. 15A). In both cell lines, treatment with cisplatin increased the apoptotic fraction of cells in control as well as myrAKT3 cells. However, the subG1 fraction was reduced in both cell lines when expressing constitutively active AKT3.

5.2.4 Downstream effectors of AKT mediated cisplatin resistance

As activation of AKT seemed to play a major role in the MEK inhibitor induced protection from genotoxically induced apoptosis, putative downstream mediators of this increase in survival were investigated.

To this end, expression of several FOXO downstream targets known to be implicated in cell cycle regulation or apoptosis was investigated in response to cisplatin and PD184352 treatment in A375 cells (fig. 16).

Cyclin D1 (*CCND1*), Cyclin B1 (*CCNB1*) as well as NOXA (*PMAIP1*) turned out to be direct targets of the ERK1/2 pathway, being downregulated after MEK inhibition. Among these three genes, cisplatin solely induced *PMAIP1*, while *CCNB1* was downregulated. In contrast, BIM (*BCL2L11*) was upregulated by PD184352 and cisplatin to a low extent but was more strongly induced in the co-treatment situation. TRAIL (*TNFSF10*) and PUMA (*BBC3*) were the only tested genes that were strongly induced by cisplatin (13 and 68 fold, respectively). Both genes, however, showed clearly reduced expression levels in presence of cisplatin and PD184352 compared to cisplatin mono-treatment.

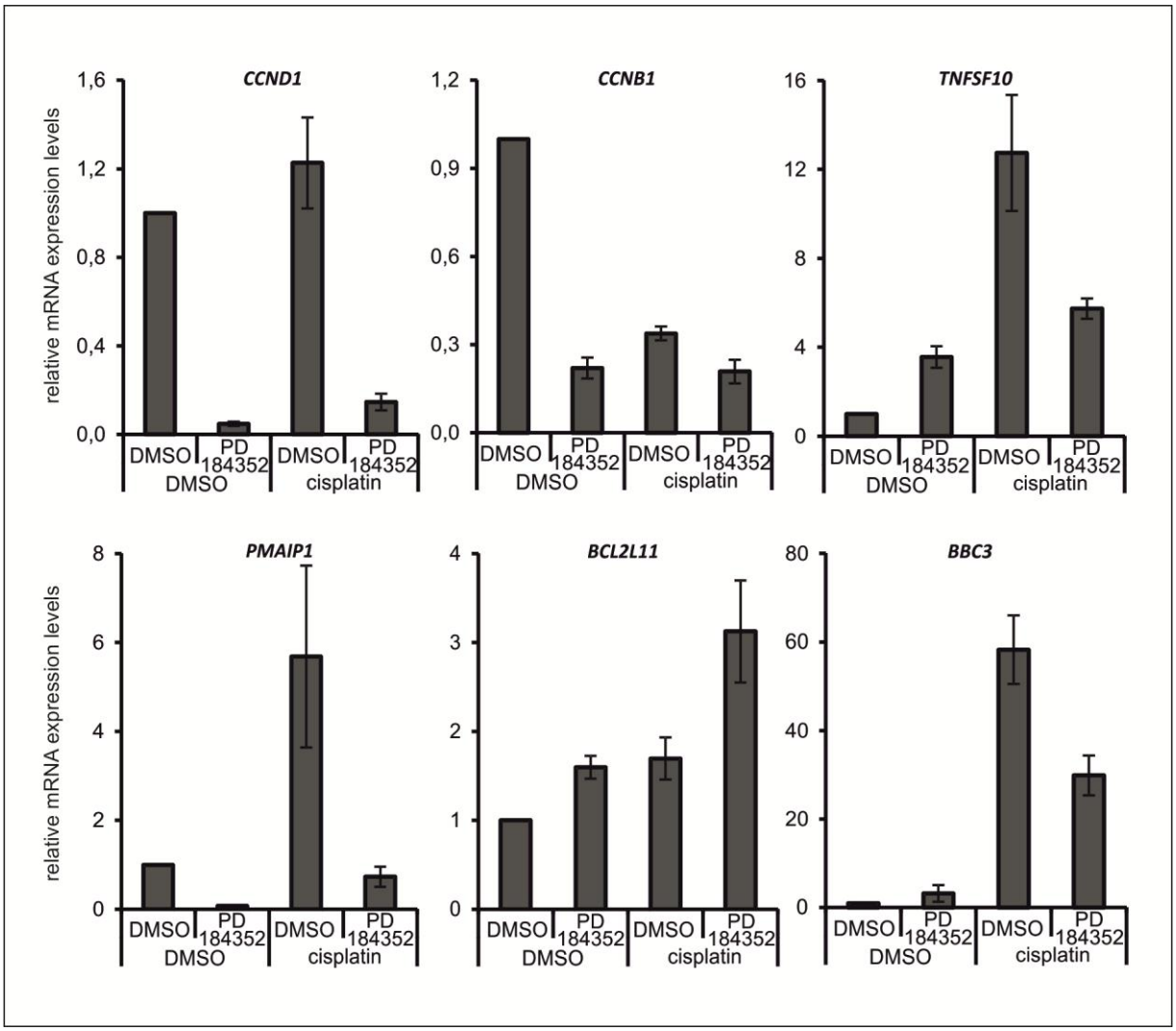

Figure 16: Real-time PCR Analysis of FOXO dependent apoptosis mediators in A375 cells. Cells were treated with cisplatin (10µM) and PD184352 (2µM) for 24h. Expression levels of *CCND1*, *CCNB1*, *TNFSF10*, *PMAIP1*, *BCL2L11* and *BBC3* were determined.

5.2.4.1 PUMA suppression mimics AKT mediated cisplatin resistance

We decided to focus on PUMA, since the induction of *BBC3* after cisplatin treatment was much higher in comparison to *TNFSF10*. Furthermore, PUMA is known to be an important mediator of the intrinsic apoptosis machinery. In A375 as well as LOX IMVI cells, treatment with cisplatin induced the expression of PUMA and thus increased its protein levels (fig. 17A). This increase was strongly reduced if PD184352 was present additionally. The same attenuating effect could also be seen in myrAKT3 expressing cells (fig. 17B). Here, constitutive activation of AKT signaling was sufficient to reduce cisplatin induced upregulation of PUMA.

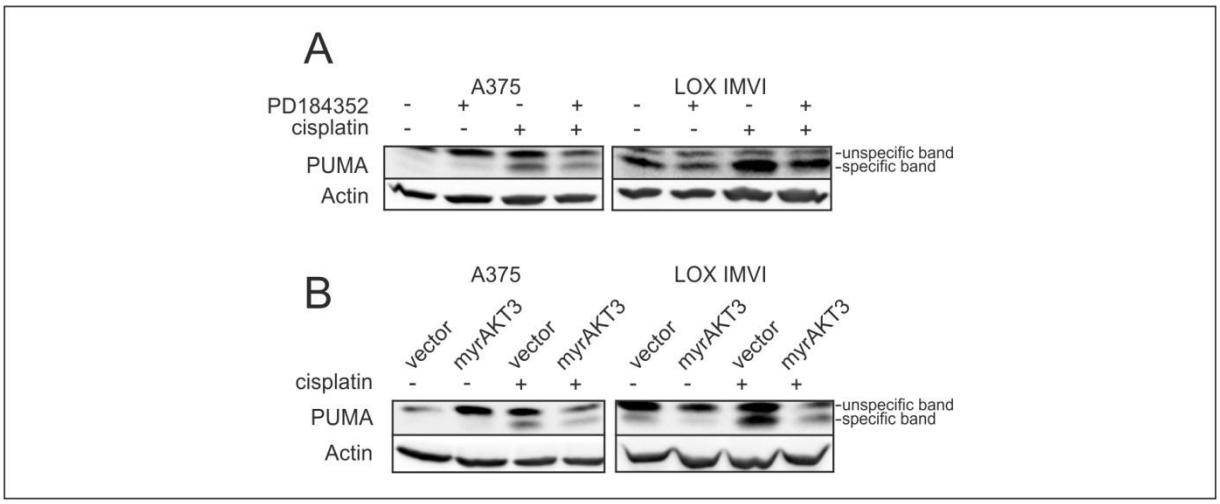

Figure 17: Cisplatin induces *PUMA* in an MEK inhibition and AKT dependent manner in A375 and LOX IMVI cells **A.** PUMA protein levels after treatment with cisplatin (10µM) and PD184352 (2µM) for 24h. β-actin served as loading control. **B.** PUMA protein levels in myrAKT3 or control vector expressing cells after cisplatin (10µM) treatment for 24h. β-actin served as loading control.

To analyze the role of PUMA in DNA damage induced apoptosis and to investigate its potential function in mediating the observed protective effect, an siRNA knockdown of *PUMA* was performed.

In A375 as well as LOX IMVI cells, cisplatin induced *PUMA* mRNA as well as protein levels could be reduced to approximately 30% in comparison to control siRNA treated cells (fig. 18A and B). This reduction of PUMA effectively reduced the levels of apoptosis after cisplatin treatment as assayed by determination of cleaved caspase3 levels (fig. 18C). Interestingly, equal amounts of P-p53 (Ser15) in knockdown as well as control cells indicate comparable levels of DNA damage, confirming the role of PUMA as a downstream apoptosis mediator. As a second marker for reduced induction of apoptosis in PUMA knockdown cells, the rates of apoptotic cells after cisplatin treatment were determined (fig. 18D and E). In A375 and LOX IMVI cells the fractions of cells in sub G1 were reduced in the *PUMA* knockdown situation compared to the controls.

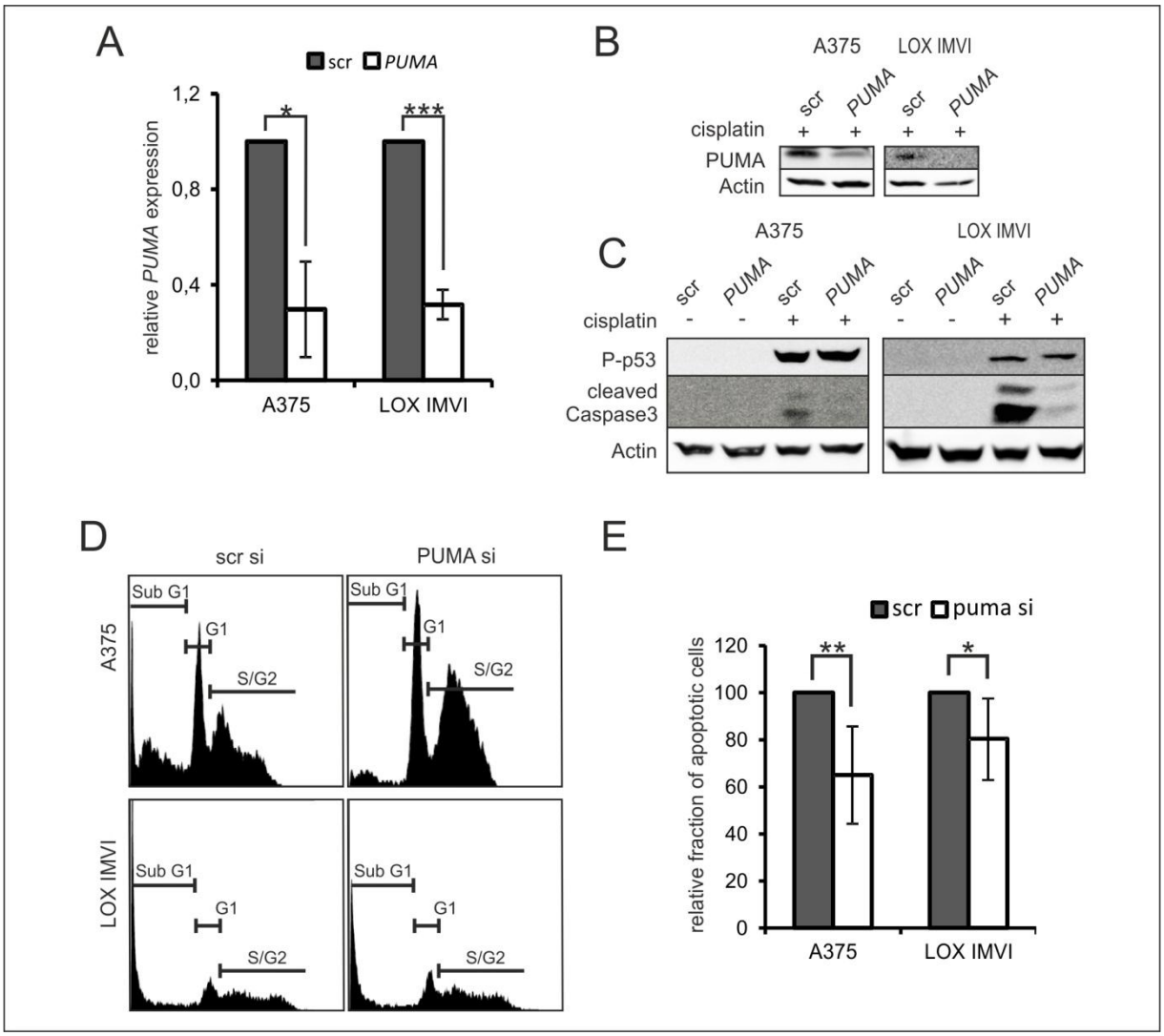

Figure 18: *PUMA* knockdown prevents from cisplatin induced apoptosis in A375 and LOX IMVI cells **A.** Relative *PUMA* mRNA expression levels in cells treated with *PUMA*-specific or scr siRNA after treatment with cisplatin (10µM) for 24h **B.** Western blot analysis of PUMA protein levels in *PUMA* knockdown and control cells after treatment with cisplatin (10µM) for 24h. β-actin served as loading control. **C.** Western blot analysis of cleaved caspase3 and P-p53 in *PUMA* knockdown and control cells after cisplatin treatment. β-actin served as loading control. **D.** Cell cycle analysis of PUMA knockdown and control cells. Cells and supernatant containing detached cells were collected after 48h of cisplatin (10µM) treatment. DNA was stained using PI. Cell-cycle profiles were determined using a flow-cytometer. **E.** Relative fraction of apoptotic cells in cells treated with *PUMA*-specific or scr siRNA. Quantification of D.

5.2.5 Upstream mediators

As described earlier, the crosstalk mediated activation of AKT occurs in a PI3K dependent manner. To get insight into the mechanism leading to PI3K/AKT activation, several possible signal mechanisms were analyzed.

At first a possible contribution of reactive oxygen species (ROS) to the crosstalk signaling mechanism was addressed. ROS had been described to be important mediators of cellular signaling, known to be able to enhance RTK dependent signal

transduction [138]. Furthermore, ERK1/2 signaling regulates various antioxidant enzymes [139] [140] [141]. Thus, MEK inhibition is likely to enhance intracellular ROS levels, which in turn could influence paralleling pathways like the AKT pathway. The different scavengers or antioxidants N-acetylcysteine (NAC), Tiron or Vitamin E (Vit E), were used to abolish ROS signaling. However, in A375 cells no consistent effect on increased P-AKT levels after PD184352 treatment for two and 48 hours could be observed (fig. 19).

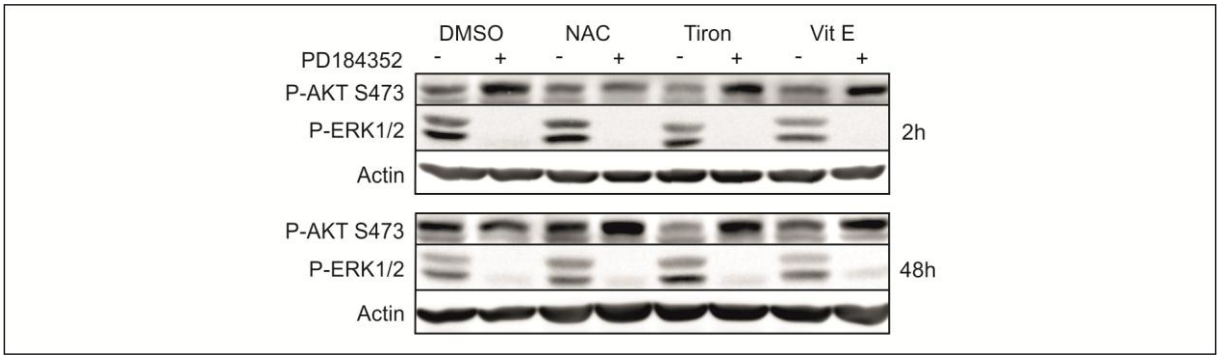

Figure 19: MEK inhibitor induced increase in P-AKT levels is not affected by ROS scavengers. Western blot analysis of A375 cells treated with PD184352 (10µM) and the ROS scavengers N-acetylcysteine (NAC, 2mM), Tiron (100µM) or Vitamin E (Vit E, 100µM)) as indicated for 2h or 48h. Levels of P-AKT (S473) and P-ERK1/2 (T202, Y204) were determined. β-actin levels served as loading control.

5.2.5.1 RTKs as mediators of AKT and ERK signaling crosstalk

Since both, the RAS/RAF/ERK pathway as well as the PI3K/AKT pathways, are downstream effector branches of receptor tyrosine kinase (RTK) signaling, RTKs might be involved in the observed pathway crosstalk.

To address whether RTKs might be involved in the observed pathway interactions and the resulting increase in chemoresistance, the expression levels of *EGF*, *IGF1*, and *PDGF* were analyzed in A375 cells. All three growth factors were reported to play a role in BRAF inhibitor resistance mechanisms in melanoma [142] [143] [117].

While *PDGF* levels were not regulated by MEK inhibition, *IGF1* expression was upregulated two fold. *EGF* was profoundly induced after PD184352 treatment. A nine-fold induction could be observed (fig. 20A). Because of this considerable EGF induction I addressed whether induction of EGFR signaling contributes to MEK inhibition induced AKT activation. Starved A375 and LOX IMVI cells were treated with PD184352 after stimulation with EGF for different timespans. Figure 20B depicts an

increasing effect of EGF stimulation on MEK inhibitor induced AKT phosphorylation levels. Thus, active EGFR enhances the crosstalk in both cell lines.

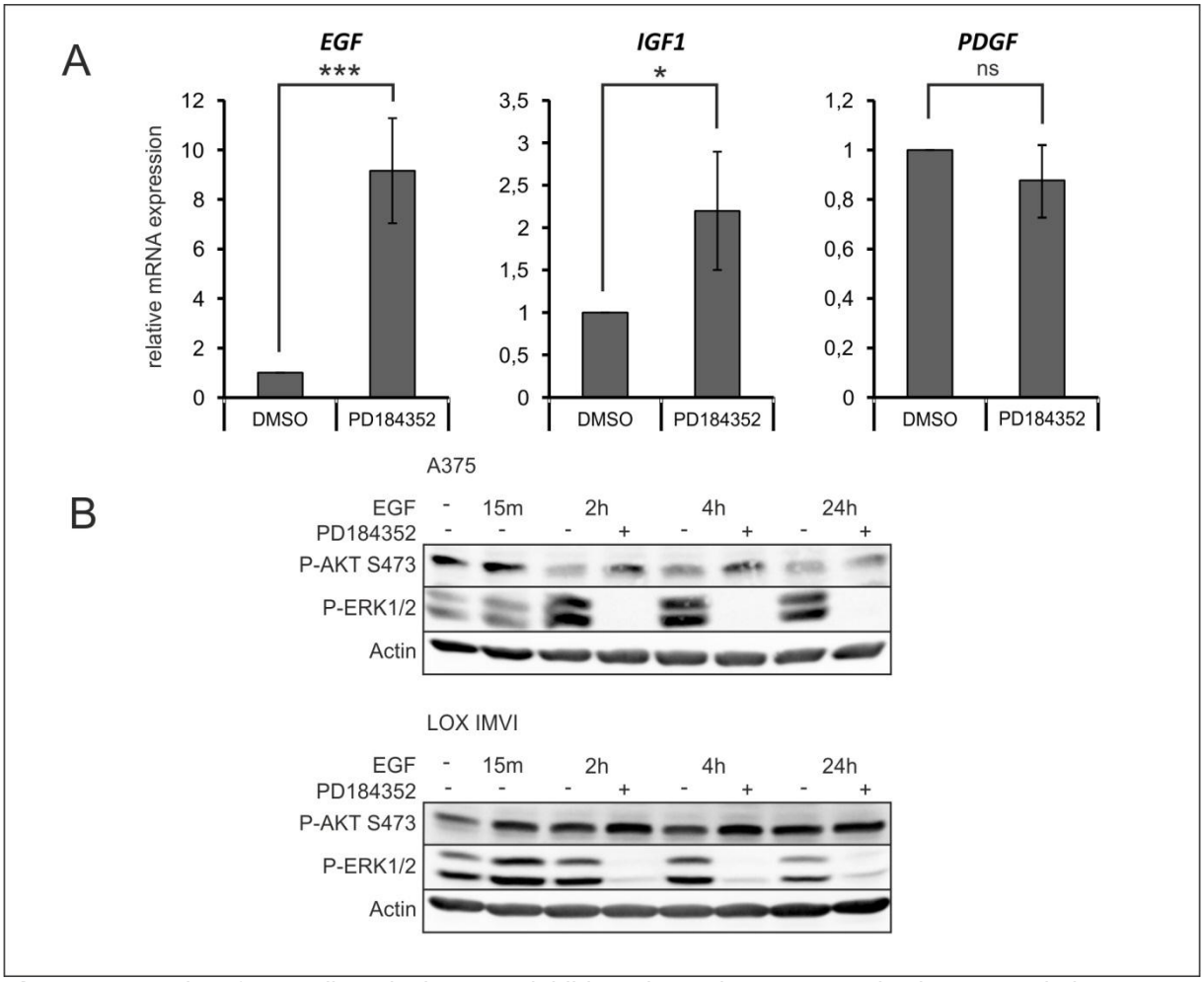

Figure 20: Role of RTK ligands in MEK inhibitor dependent AKT activation **A.** Relative mRNA expression levels of *EGF*, *IGF1*, and *PDGF* in A375 cells treated with PD184352 (2µM) for 24h **B.** Western blot analysis of A375 and LOX IMVI cells starved for 48h and treated with EGF (100ng/ml) and PD184352 (2µM) for the indicated periods. Levels of P-AKT (S473) and P-ERK1/2 (T202, Y204) were determined. Actin levels served as loading control.

To get an insight into the contribution of EGFR on the proliferation kinetics of A375 and LOX IMVI cells, the xCELLigence system was used to indirectly determine cell viabilities over a long time period and in high resolution in presence or absence of the EGFR inhibitor AG1478 (fig. 21). This method relies on impedance measurements, which basically give information about the cell density on the plate (a high impedance indicating high cell density). A375 cells showed slightly reduced growth curves in presence of the EGFR inhibitor. MEK inhibition strongly attenuated growth as expected. Application of AG1478 and PD184352 had a weak additive effect in reduction of cell growth (fig. 21A). LOX IMVI cells in contrast showed virtually no

change in growth in mock, PD184352, or AG1478 treated cells. Merely the combination of MEK and EGFR inhibitors markedly reduced cell growth (fig. 21B)

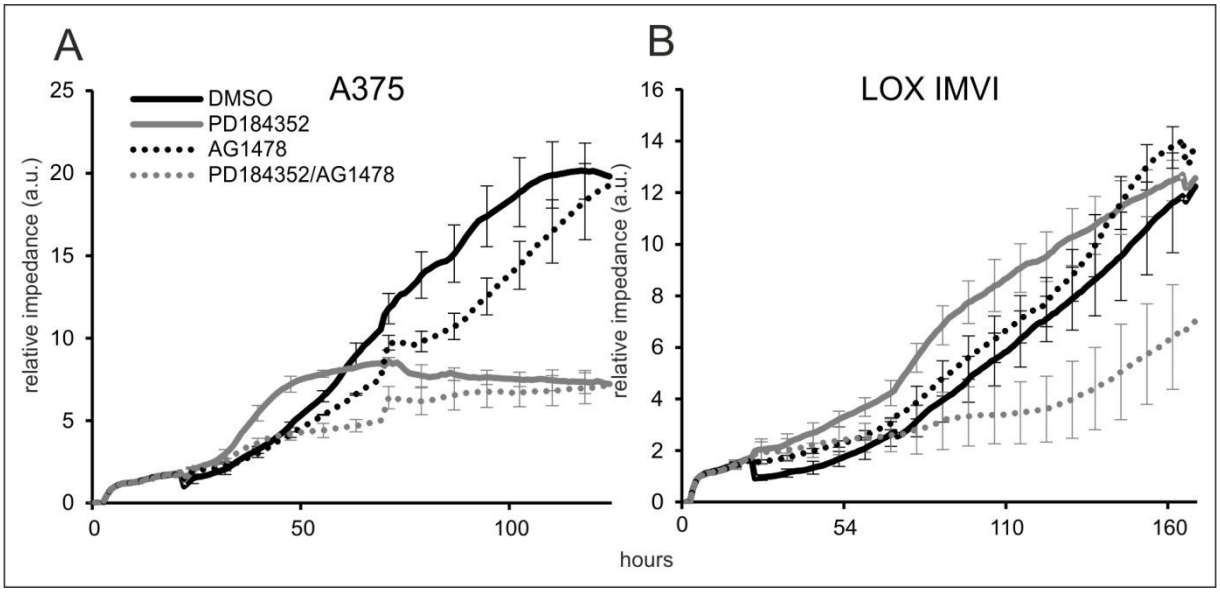

Figure 21: Effect of EGFR and MEK inhibition. A375 (**A**) and LOX IMVI (**B**) cells were incubated with DMSO, PD184352 (2µM), and AG1478 (10µM). Compounds were added 24h after experiment start. Every hour the relative impedance (arbitrary unit; a.u.) was measured using the xCELLigence system (Roche). Curves are normalized to the time-point of experimental start; to improve perceptibility only every tenth data point is displayed with error bars.

To analyze whether of RTKs in general and EGFR in particular contribute directly to the observed crosstalk resulting in the phenotypical effect of an increased survival after cisplatin treatment, A375, LOX IMVI, RPMI7951 and 451Lu cells were incubated in presence of the EGFR inhibitor AG1478 or the multi-RTK-inhibitor TKI25 as well as cisplatin and PD184352 as indicated (fig. 22).

Treatment with AG1478 alone had only a weak effect in A375 and LOX IMVI cells, while the numbers of surviving cells in RPMI7951 and 451Lu cells were strongly reduced after 4 days of treatment (fig. 22A, left). Administration of PD184352 or PD184352 together with AG1478 had a strong growth reducing effects in all tested cell lines except RPMI7951. In contrast, TKI258 mono-treatment showed only a marginal if any growth inhibitory effect in all tested cell lines (fig. 22B, left). Additional administration of TKI258 together with PD184352 increased the growth reducing effect of the MEK inhibitor only in LOX IMVI cells to a higher extent.

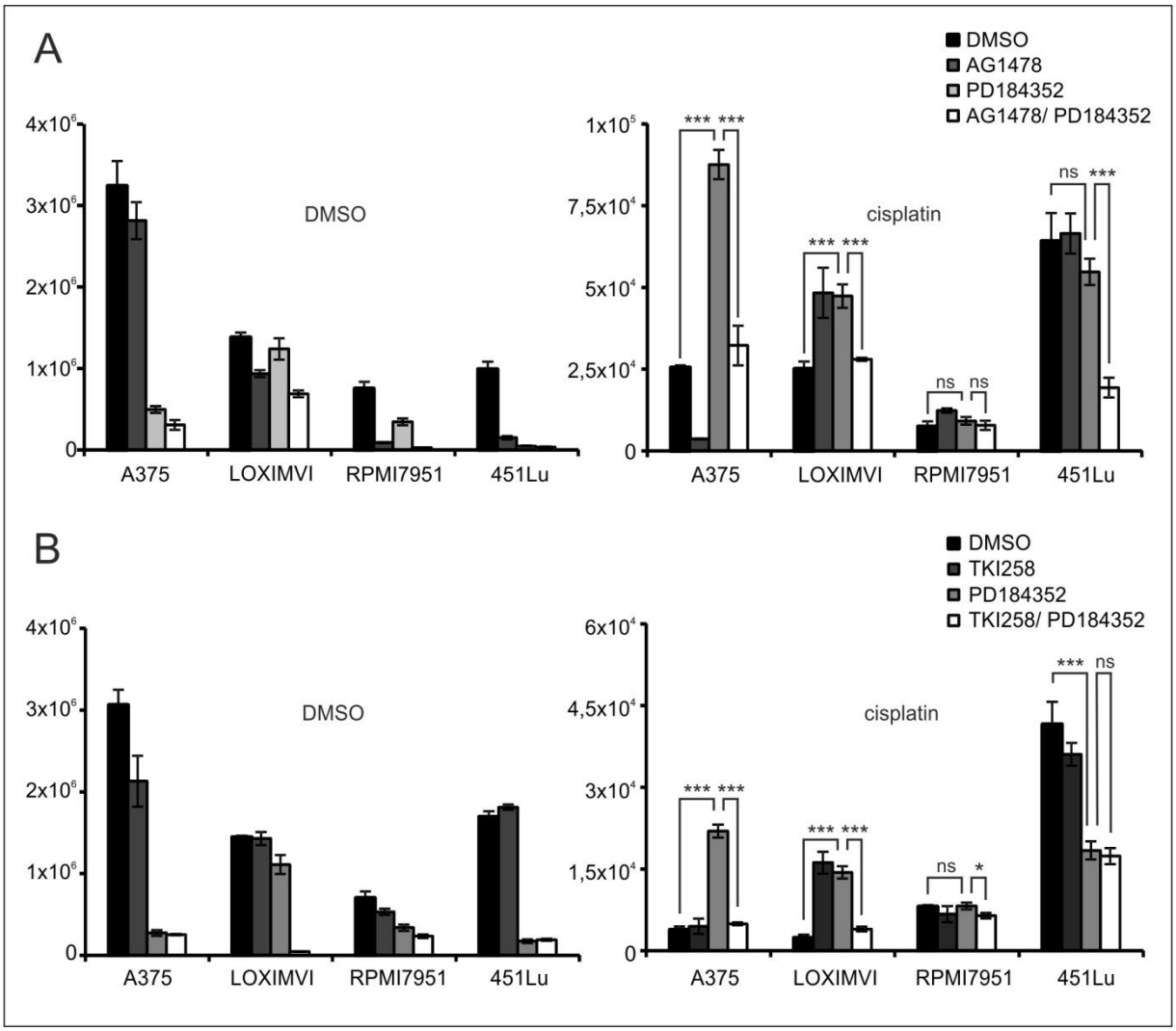

Figure 22: Role of RTKs in MEK inhibitor dependent increased survival towards cisplatin treatment. A375, LOX IMVI, RPMI7951, and 451Lu cells were treated for 96h with DMSO, PD184352 (2µM), AG1478 (10µM, **A**) or TKI258 (10µM, **B**) in presence of either DMSO (**left**) or cisplatin (10µM, **right**). Cells numbers were determined manually.

In presence of cisplatin (fig. 22A and B, right), however, in A375 and LOXI MVI cells the protective effect mediated by MEK inhibition was abolished by co-incubation with both RTK inhibitors. Interestingly, mono-application of either RTK inhibitor in presence of cisplatin led to an increased survival of LOX IMVI cells. In RPMI7951 no change in survival was observed after additional treatment with any inhibitor combination, while 451Lu cells were characterized by a high susceptibility towards all used drugs which showed additive effects in their ability to reduce the numbers of remaining cells.

5.2.5.2 SPRY involvement

Since the ERK1/2 pathway is tightly regulated by feedback mechanisms and inhibition of MEK strongly interferes with signaling output we analyzed whether the Sprouty family of feedback regulators might be implicated in mediating the observed increase in phosphorylation of AKT after MEK inhibition. Since members of the Sprouty family have been described to be able to interact with upstream signaling components that are not exclusively RAF/MEK/ERK pathway mediators but are also able to contribute to PI3K/AKT pathway regulation [76], [79–81], [83], an effect seemed to be probable.

As a starting point, the expression levels of *SPRY1-4* and *SPRED1* and *2* were analyzed as a function of time after MEK inhibition using PD184352. As depicted in figure 23, inhibition of MEK strongly reduced the expression levels of *SPRY1, 2* and *4* as well as *SPRED1* and *2*. The reduction of *SPRY2* mRNA levels was weaker in comparison to the reduction observed for the other Sprouty members. *SPRY1*, *SPRED1* and *2* levels remained unchanged after one hour of PD184352 treatment. *SPRY3* expression in contrast was independent of RAS/RAF/ERK signaling output. To further investigate the MEK dependent regulation of SPRY1, 2 and 4, the protein levels were determined in primary melanocytes (NHEM) as well as A375 and LOX IMVI cells that were incubated in presence or absence of PD184352 for 24 hours (fig. 23B). The primary melanocytes showed much lower levels of all Sprouty proteins in comparison to the established melanoma cell lines, although they displayed high levels of activated P-ERK1/2. In A375 and LOX IMVI cells a considerable reduction of protein levels could only be observed in case of SPRY4. SPRY1 and 2 levels were not or only weakly reduced. In A375 cells, SPRY1 levels seem to be completely unaffected by MEK inhibition.

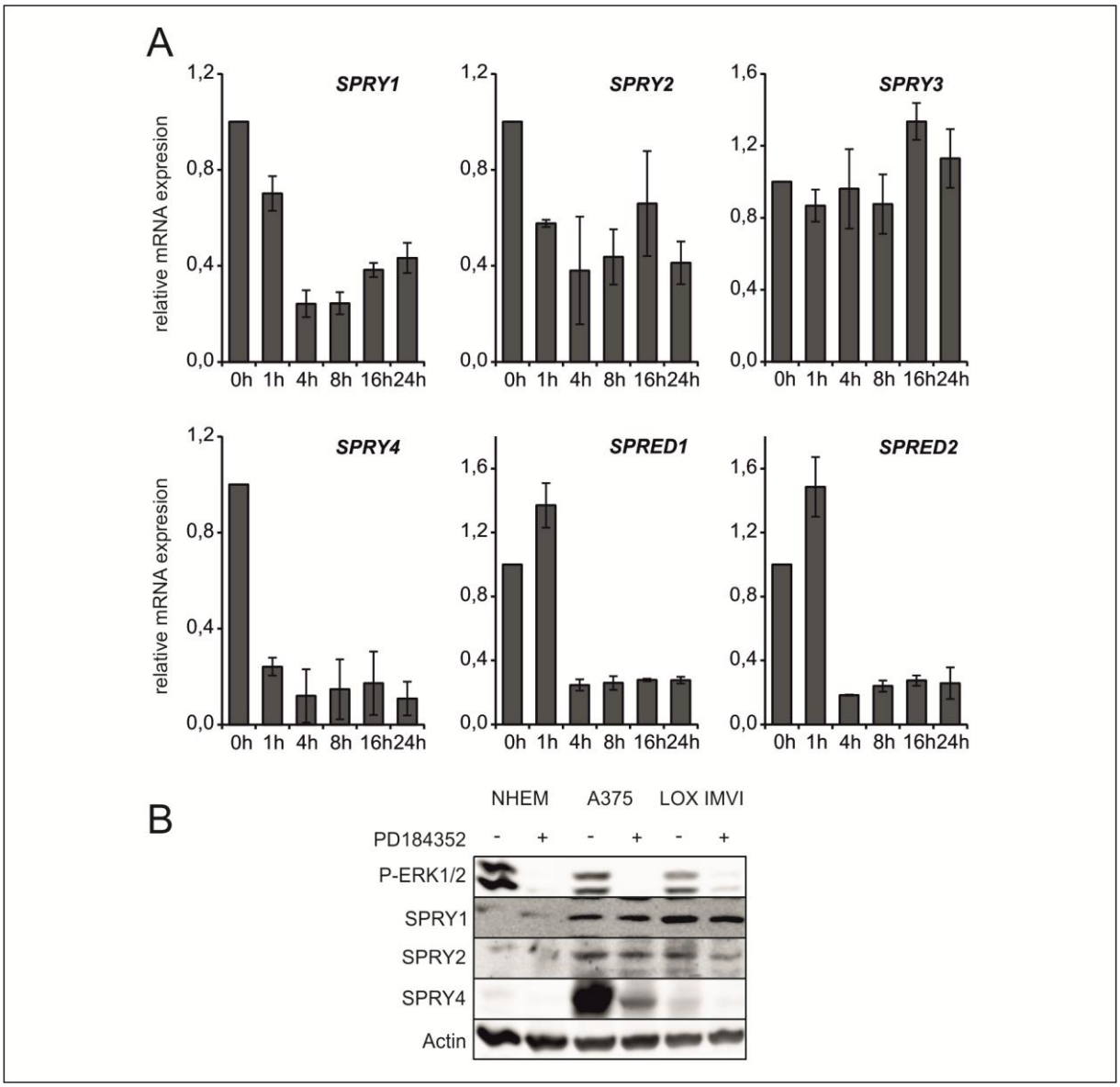

Figure 23: SPROUTY family members after MEK inhibition **A.** Relative mRNA expression levels of *SPRY1-4* and *SPRED1,2* in A375 cells treated with PD184352 (2µM) for the indicated periods. Normalized to untreated cells (0h) **B.** Western blot analysis of SPRY1,2,4 in NHEM, A375, and LOX IMVI treated with PD184352 for 24h. P-ERK1/2 levels were determined as inhibitor control, Actin served as loading control.

To get an insight whether Sprouty protein mediated feedback regulation might be implicated in the observed MEK/ERK1/2- PI3K/AKT crosstalk, transfections of specific siRNAs directed against *SPRY1, 2* or *4* were conducted. The knockdown could be verified on the mRNA level (fig. 24A). As shown for the cell line A375, *SPRY1* siRNA had the highest efficiency, reducing *SPRY1* transcript levels to 34%. *SPRY2* and *4* knockdowns did not reach such efficient levels. The corresponding transcript levels were reduced to 64% and 62% respectively. Unfortunately, on the level of protein the knockdown could only be confirmed for SPRY4. No difference in protein abundance could be detected after knockdown of *SPRY1* and *2* (fig. 24B). In

addition to that, neither knockdown had any influence on the phosphorylation levels of ERK1/2 or AKT 48 hours after siRNA transfection. Still, Sprouty knockdown cells were incubated in presence of cisplatin for 48h starting 48h after siRNA transfection. As apparent in figure 24C, no differential induction of cleaved caspase3 after genotoxic stress could be observed in control siRNA transfected cells in comparison to cells transfected with *SPRY1, 2* or *3* siRNA. Cells that were kept without cisplatin being present showed no induction of cleaved caspase3 (data not shown). Infliction of equal amounts of DNA damage was ensured by confirmation of equal levels of phosphorylated p53. To further analyze whether there might be a cumulative effect on cell survival after 48 hours of cisplatin treatment, the numbers of surviving siRNA treated cells were determined (fig. 24D). Neither A375 nor LOX IMVI cells showed any significant differences in the number of viable cells.

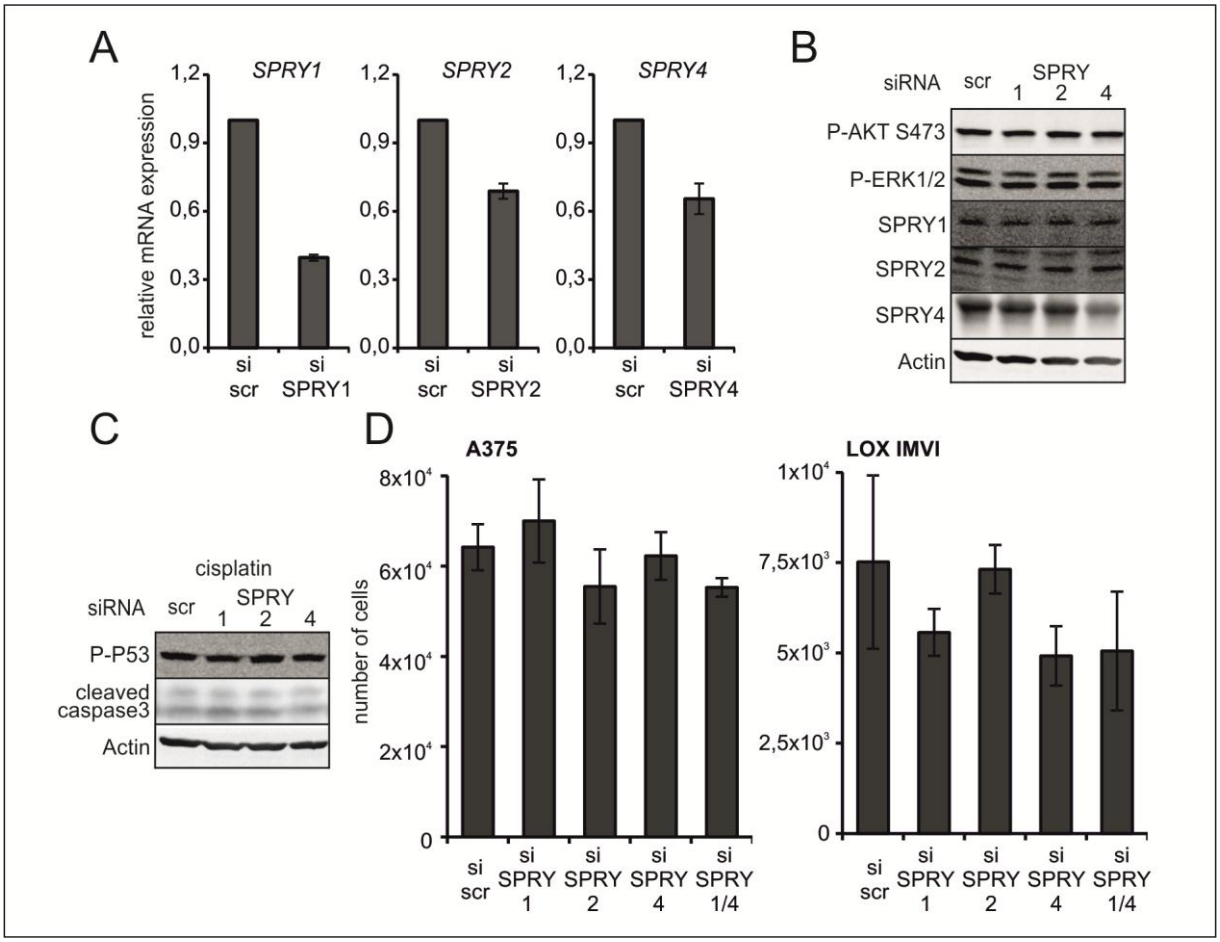

Figure 24: Effects of siRNA mediated knockdown of *SPRY1,2,4* **A.** Relative mRNA expression levels of *SPRY1-4* in A375 cells transfected with *SPRY1,2,4* specific siRNA or scr siRNA after 48h. **B.** Western blot analysis of SPRY1,2,4, P-ERK1/2 (T202, Y204), P-AKT (S473), and Actin in A375 cells 48h after transfection with *SPRY1,2,4* specific siRNA or scr siRNA. **C.** Western blot analysis of P-P53 and cleaved caspase3 in siRNA transfected A375 cells in presence of cisplatin (10µM). Actin served as loading control. **D.** Number of living cells in siRNA transfected A375 (**left**) and LOX IMVI (**right**) cells. 48h after transfection cells were treated for 48h with cisplatin (10µM). Cell number was determined manually.

To rule out that the results obtained by the knockdown experiments were not due to insufficiently low knockdown efficiencies, dominant negative versions of SPRY2 and SPRY4 (SPRY2dn; SPRY4dn) were used in a second approach. For SPRY1 no information on a dominant negative version was available. Both dominant negative protein versions had been described to act in a dominant negative manner not only towards the corresponding wildtype SPRY but also towards the wildtype form of each other [133].

Overexpression of the myc-tagged variants was performed in A375 cells. The presence of the overexpressed dominant negative versions could be verified by detecting the myc-tag (fig. 25A).

In contrast to SPRY2dn, SPRY4dn was able to increase phosphorylation levels of ERK1/2 and AKT to a certain extent. Cisplatin treatment induced levels of cleaved caspase3. This induction, however, occurred not differently in control cells or cells transfected with any dominant negative SPRY tested. Confirming those results, cell cycle analyses of cisplatin treated cells expressing SPRY2dn or SPRY4dn, showed only marginal changes in numbers of cells accumulating in the apoptotic SubG1 fraction like control cells (fig. 25B). To investigate whether different members of the Sprouty family are able to rescue the loss of another family member as it has been shown to be the case in a different scenario [74], combinations of overexpressed dominant negative versions with siRNA knockdown of the remaining MEK dependent regulated SPRYs were conducted. In the cell line A375, no differential phosphorylation levels could be observed in cells deprived of functional SPRY1, 2 and 4 (fig. 25C). Still, effects on proliferation and chemoresistance could possibly occur in spite of the undetectable signaling molecule activation. Therefore, the number of living cells after 48h of growth in presence or absence of cisplatin was determined (fig. 25D). A slight increase in proliferation could be observed in SPRY2dn cells when transfected with either siRNA mixture as indicated. Only a minor change in cell survival in presence of cisplatin could be observed. The increased numbers of surviving SPRY2dn expressing cells in presence of cisplatin mirror the increase of overall proliferation in the DMSO treated cells.

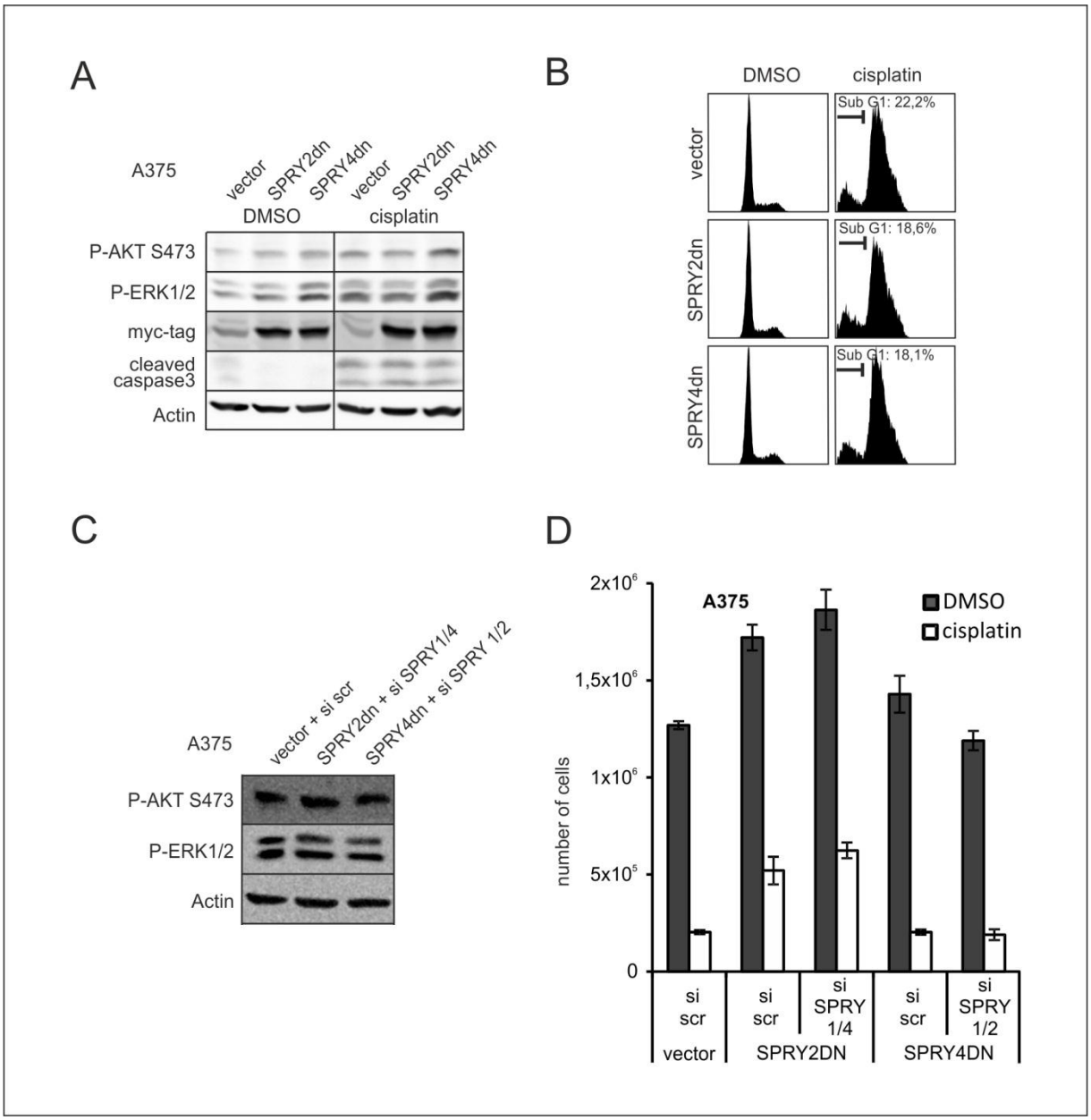

Figure 25: Effects of dominant negative SPRY2 and SPRY4 and *SPRY1, 2, 3* siRNA. **A:** Western blot analysis of P-ERK1/2 (T202, Y204), P-AKT (S473), and cleaved caspase3 in A375 cells stably transfected with SPRY2dn, SPRY4dn or vector control and treated with DMSO or cisplatin (10µM) for 24h. Detection of myc-tag and β-actin served as infection and loading control. **B:** Transfected cells and their supernatant (containing detached cells) were collected. DNA was stained using PI. Cell cycle profiles were compiled using a flow cytometer. **C.** Western blot analysis of A375 cells stably transfected with SPRY2dn or SPRY4dn or vector control. Additionally siRNA mediated knockdown of the remaining SPROUTY members was performed. As control, cells containing the empty vector were transfected with scr siRNA. Levels of P-AKT (S473) and P-ERK1/2 (T202, Y204) in presence of cisplatin (10µM) were determined 3 days after transfection. ß-actin served as loading control. **D.** Number of living cells in siRNA transfected A375 cells carrying SPRY2dn or SPRY4dn. 48h after transfection cells were treated for 48h with cisplatin (10µM). Cell number was determined manually.

Taken together, a contribution of RTK signaling and specifically EGFR signaling in the ERK/AKT crosstalk is likely. However, only a slight contribution of SPRY proteins could be identified in the assayed setting of melanoma AKT/ERK signaling crosstalk.

6 Discussion

Research on basic signaling mechanisms as well as drug interaction mechanisms is an essential step in understanding the complex processes during melanomagenesis. It constitutes not only the basis for the development of enhanced therapeutic approaches but might also help in establishing suitable melanoma classifications. To this end I used not only human melanoma cell lines but also the HERmrk model.

6.1 Functional validation of Xmrk targets in the HERmrk model and in human melanoma

The Xmrk receptor tyrosine kinase, being a very well established melanoma oncogene in the *Xiphophorus* melanoma model, proved to be a suitable oncogene in a transgenic murine melanocyte cell culture model, recapitulating aspects of melanomagenesis as seen in human melanoma [52], [54], [55]. In particular, the activation of common melanoma associated signaling pathways [52], as well as similarities in gene expression profiles [55] in comparison to human melanoma systems driven by oncogenic BRAF [68], [144], underscore the comparability of the Xmrk model and the human disease. *Fosl1* and *Mkp2* were identified as transcriptional targets of Xmrk using a microarray based approach [55] and were further analyzed and validated as potential mediators in human melanomagenesis.

6.1.1 Fosl1

Similar to the situation in the Xmrk model, *FOSL1* upregulation could also be observed in a variety of melanoma cell lines if compared to primary NHEM cells. Interestingly, the mRNA levels turned out to be increased in cell clones derived from metastatic sites (A375M, LT5.1) compared to their isogenic counterparts obtained from the primary tumor (A375, Dx3) [55]. For the A375/A375M pair this could also be seen on the protein level. FOSL1 is a member of the AP-1 complex, being able to dimerize with JUN family members [135] [136]. The AP-1 complex has been linked to epithelial mesenchymal transition (EMT) as well as invasion, motility and transformation in various cancer types (reviewed in [147] [148]). Thus it might well be that FOSL1 is an important factor of AP-1 function with regard to metastatic potential.

Indeed, several other studies have shown an explicit function of FOSL1 during these processes [149] [150] [151] [152] [153] [154]. Interestingly, in melanoma, FOSL1 protein levels turned out to be highly variable in their abundance in different melanoma cell lines indicating the possibility of a context dependent expression that might be influenced by the cell line`s origin. The observed high heterogeneity of melanoma samples and cell lines has been described earlier and is considered to be a known feature of melanoma [155]. In further analyses it might be promising to analyze whether there is a common correlation of FOSL1 abundance with cell line origin (primary site or metastasis) or a difference in isogenic cells after consecutive xenograft colonization in mice. First results obtained from immunohistochemically stained tissue samples derived from melanoma patients show a markedly increase of FOSL1 abundance from nevi stages to primary tumor tissue and late melanoma metastases (unpublished data; Bosserhoff, Meierjohann). The same pattern of FOSL1 levels has been described in breast cancer, where FOSL1 was shown to be more abundant in carcinomas compared to early hyperplastic stages [156].

Comparable to other RTKs [157] like EGFR, Xmrk is able to trigger several paralleling signaling pathways [52]. FOSL1 expression turned out to be induced in an ERK1/2 dependent manner in melanoma as shown by the disappearance of mRNA as well as protein after MEK inhibition. This dependence could be shown in the murine melanocyte model as well as in human melanoma cell lines and is in accordance with previously published data obtained in RAF or RAS transformed fibroblast or kidney cell lines, where FOSL1 expression was shown to be ERK1/2 dependent as well [158] [159]. In addition to that, there is evidence that also the protein stability of FOSL1 is increased after ERK1/2 dependent phosphorylation at specific sites [158] [160].

The knockdown of FOSL1 was performed in two cell lines carrying $BRAF^{V600E}$, eliminating potentially differences derived from different melanoma oncogenes. A375 cells are characterized by high FOSL1 levels and Mel Ho cells have low levels of FOSL1. Both cell lines showed a slightly reduced proliferation rate as assayed by BrdU incorporation after *FOSL1* knockdown. Regulation of cell growth has already been connected to AP-1 function [148]. Cyclin D1, which is required to enter G1 phase during the cell cycle is a prominent regulator of cell growth. It was shown to be regulated by FOSL1 in breast cancer cells as well as alveolar cells [153] [161]. Even more interestingly, in the light of the pivotal role of AP-1 in the complex event of

metastasis, in Mel Ho cells, the knockdown reduced the cells` migratory potential [55]. Consequently, there is an established set of FOSL1-dependent genes, which is implicated in migration as well as invasion. The receptor tyrosine kinase AXL was shown to be regulated in a FOSL1 dependent manner in bladder cancer. In this model, AXL mediates the effects of FOSL1 on migration and motility [150]. Besides AXL, MMP1, MMP9, TIMP1, MET or CD44 have been described as FOSL1 dependent mediators of migration [152] [153] [162].

Taking together, increased levels of FOSL1 in melanoma are regulated by ERK1/2 signaling output and have a pro-proliferatory and pro-migratory effect. Thus, FOSL1 so far not considered, may become a useful prognostic marker for melanoma invasiveness.

6.1.2 MKP2

To find out if MKP2 is of functional importance in Xmrk induced oncogenic signaling, knockdown experiments were performed in HERmrk^{hi} and HERmrk^{me} cells. Both cell lines are derived from the murine melanocyte cell line melan-a, and are stably transfected with HERmrk, the EGF-activatable variant of Xmrk. However, the expression levels of the oncogene are much higher in the HERmrk^{hi} clone in comparison to HERmrk^{me}. This causes differences in signaling strength and leads to different phenotypes. After induction of Xmrk signaling, HERmrk^{hi} cells are primarily driven into senescence, while HERmrk^{me} cells are characterized by a strongly enhanced proliferation [54]. This dependence on signaling strength renders MKP2 a potentially interesting factor in Xmrk induced activation of ERK1/2 signaling in this system, since its role in attenuating the signaling output of this pathway is well established [61].

The knockdown of *Mkp2* in HERmrk^{me} cells had no measurable effect at all. In contrast, proliferation of HERmrk^{hi} cells was strongly blocked in response to *Mkp2* knockdown. Unexpectedly, this went along with reduced levels of active ERK1/2, which prevented the induction of *Opn* and *Egr1*, both being ERK1/2 dependent target genes of HERmrk signaling [55].

The effects of the *Mkp2* knockdown in HERmrk^{hi} cells cannot be explained by the loss of the ERK1/2 phosphatase function of MKP2, which would lead to an increase in P-ERK1/2 levels. The observed reduction of ERK1/2 signal, target gene

expression and proliferation might be caused by the effects of additional MKP2 targets. A reduction of ERK1/2 phosphorylation has been described after the loss of the closely related MKP-family member PAC-1 (DUSP2). In immune cells, PAC-1 loss caused the reduction of P-ERK1/2 levels by increasing the activity of JNK. [163]. Since both, PAC-1 and MKP2 are able to dephosphorylate JNK, a similar mechanism is probable. In lung cancer cell lines that are dependent on EGFR, knockdown of MKP2 also resulted in reduced ERK1/2 phosphorylation [164]. There is, however, contradicting data derived from lung adenocarcinoma cells. Here, EGFR dependence is accompanied by MKP2 loss [165]. Additionally, it has been described that loss of functional MKP2 can have a growth attenuating effect independent of ERK signaling output. In multiple endocrine neoplasia, driven by the receptor tyrosine kinase RET, inhibition of MKP2 inhibited proliferation, while its effects on ERK1/2 phosphorylation were only marginal [134]. Regulation of cyclin B levels were found to be MKP2 dependent in this multiple endocrine neoplasia model [134] as well as in murine MKP2$^{-/-}$ embryonic fibroblasts (MEFs) [71]. Thus, there are additional modes of action for MKP2 in other malignancies, which could explain the observed phenotypes and may play a role in the melanoma system as well.

In contrast to that, previously described MKP dependent interference in the induction of senescence has been shown to be mediated by ERK1/2. Replicative senescence, which is accompanied by reduced ERK1/2 signaling can be avoided by knocking down MKP2. [166] [167] [168]. However, since the primary effect of the *Mkp2* knockdown in HERmrk^{hi} cells is quiescence (see the strongly reduced proliferation in the starved situation, figure 4D), it is not possible to evaluate effects on senescence in this cellular setting.

Comparing the results obtained in HERmrk^{me} and HERmrk^{hi} cells it becomes obvious that the basic signaling strength caused by different HERmrk expression levels is fundamental when analyzing signaling modifiers like MKP2. A similar conclusion can be drawn from the contradicting results obtained from human melanoma cell lines. Knockdown and overexpression had differential effects on two distinct cell lines. A375 cells, known to express low levels of MKP2 [55] were sensitive to a further knockdown, which resulted in reduced proliferation rates, a situation comparable to HERmrk^{hi} cells and MKP2$^{-/-}$ MEFs [71]. Overexpression of MKP2 only weakly enhanced the proliferation in this cell line. In contrast, in Mel Ho cells, having MKP2 in much higher abundance than A375 cells [55], the knockdown had no effect, while

the overexpression strongly enhanced cell growth. Interestingly, the closely related MKP3 had similar diverse effects on tumorigenicity in different melanoma models in humans and mice [69].

Taken together, the functional role of MKP2 seems to be highly diverse in different cellular settings. Different oncogenes, signaling strengths, protein abundances and further cellular circumstances contribute to a plethora of different modes of MKP2 function. Furthermore, additional targets besides ERK1/2 and thus additional pathway interactions are very likely to be important to understand MKP2 function. However, the MKP2 status might well be an important factor for a thorough classification of melanoma.

6.2 MEK inhibition prevents cisplatin induced apoptosis in a AKT and PUMA dependent manner

MEK inhibition in mono-application is currently a well pursued treatment strategy for metastatic melanoma showing first promising results in clinical studies [126]. Nevertheless, combination studies will be of high interest, since secondary resistances are quite likely to occur as described in other kinase inhibitor treatment regimen. For vemurafenib, which is also used to target the ERK1/2 signaling axis in melanoma, there is a growing number of described resistance mechanisms (reviewed in [169]). In addition, combination of chemotherapeutics and MEK inhibitors were shown to be a feasible approach in colorectal cancer [170]. Thus, the efficacy of MEK inhibition in combination with cisplatin treatment in melanoma will be discussed in the following.

6.2.1 Effects of MEK inhibition in melanoma cell lines are diverse

The consequences of MEK inhibition in melanoma cells proved to be quite diverse within the panel of tested melanoma cell lines. As it was shown earlier, MEK inhibition is able to induce apoptosis [171] [172] and an accumulation of cells in G1, resulting in a reduction of proliferation rates [173] [174]. All tested cell lines showed both apoptosis and growth arrest, but the ratios varied strongly. LOX IMVI, A375, RPMI7951, and MeWo cells displayed some kind of intrinsic resistance towards the apoptosis inducing effect of MEK inhibition and were predominantly characterized by G1 arrest. The source of this resistance, however, might be diverse among the different cell lines. RPMI7951 cells, for example, have previously been shown to express high levels of the RAS/RAF/ERK1/2 pathway agonist COT. This was shown not only to confer intrinsic resistance towards $BRAF^{V600E}$ but also towards MEK inhibition [120]. However, the described elevation of ERK1/2 phosphorylation despite presence of MAPK pathway inhibitors could not be verified in my experiments, emphasizing the possibility of additional mechanisms involved in mediating MEK inhibitor resistance. MeWo cells in contrast to the other three cell lines do not carry oncogenic BRAF. This, however, was shown to be a criterion for MEK inhibitor susceptibility in vitro [124] and in vivo [125]. Resistance mechanisms in A375, characterized by the by far highest intrinsic proliferation rate, and LOX IMVI cells,

displaying the highest levels of resistance even at MEK inhibitor concentrations up to 4µM, remain a matter of speculation. However, it is important to mention that a weak but measurable level of phosphorylated ERK1/2 was detected in these two cell lines 48h after application of the MEK inhibitor. Reactivation of ERK signaling has been identified to contribute to resistance mechanisms towards MEK inhibition in several cancer types [175] and in particular after $BRAF^{V600E}$ inhibition in melanoma [120] [116]. In these situations, however, ERK1/2 phosphorylation levels were considerably higher.

I could show that a common feature for all cell lines characterized by low MEK inhibitor susceptibility was a treatment-induced phosphorylation of AKT. This was most prominent in A375, LOX IMVI as well as RPMI7951 cells and might possibly contribute to the low susceptibility towards PD184352, as AKT activation has already been linked to MEK inhibitor resistance mechanisms in melanoma [176] [172] and other malignancies [177] [178] [179]. Furthermore, there are several examples derived from the application of $BRAF^{V600E}$ specific inhibitors in melanoma patients that emphasize the importance of the PI3K/AKT axis in RAS/RAF/ERK1/2 pathway inhibition induced drug resistance [180] [118] [119].

In addition to the analyzed human melanoma cell lines, an upregulation of P-AKT levels was also found to be evident in HERmrk cells when treated with U0126, another well described MEK inhibitor. Furthermore, presence or absence of active RTKs (HERmrk in the murine system or any endogenously expressed RTK in the human cell lines) seemed to be important for the observed pathway crosstalk. Stimulation of RTK signaling strongly enhanced MEK inhibitor treatment induced AKT activation compared to the activation observed under starved conditions. The most explicit upregulation of P-AKT levels occurred at 2 hours after MEK inhibitor treatment while treatment for 30 min was not sufficient to induce AKT phosphorylation. Such a crosstalk kinetic hints at a possible contribution of transcriptionally regulated factors, as discussed below.

6.2.2 MEK inhibitor induced chemoresistance is mediated via AKT

The effect of MEK inhibitor and cisplatin co-treatment was expected to be additive in the induction of apoptosis as this has been proposed earlier in comparable scenarios. An active RAS/RAF/ERK1/2 signaling cascade was shown to enhance the

DNA repair capability after cisplatin treatment [181]. In melanoma it was observed previously that RAF inhibition by sorafenib enhances the effects of dacarbazine, an alkylating cytostatic drug [182]. Docetaxel, an anti-mitotic drug and the MEK inhibitor Selumetinib were described to have additive effects [174]. Furthermore, in human lung tumor xenografts the MEK inhibitor PD184352 was shown to increase the apoptotic inducing effect of paclitaxel, another drug belonging to the class of anti-mitotic compounds [183]. However, in contrast to cisplatin, docetaxel and paclitaxel do not induce DNA damage, but mitotic crisis, and thus might have different effects if combined with ERK1/2 pathway inhibition.

Cisplatin mono-application effectively induced apoptosis in all tested cell lines. In those cell lines that were characterized by apoptotic susceptibility towards MEK inhibition, cisplatin and PD184352 indeed had additive effects in the induction of cell death, as anticipated. This can be seen from the reduced numbers of surviving cells, increased rates of cells in subG1 as well as elevated levels of cleaved caspase3. Unexpectedly, additional administration of PD184352 reduced the genotoxic effect of cisplatin in A375, LOXIMVI, RPMI7951, and MeWo cells, as observed by an increased number of surviving cells, a decreased fraction of cells in subG1 cells and reduced levels of cleaved caspase3. This increase in chemo-resistance after genotoxic treatment was observed only in cell lines that were not driven into apoptosis after MEK inhibition per se, and in addition displayed AKT activation after PD184352 treatment. MeWo cells, despite having a low susceptibility towards MEK inhibition, showed no AKT activation and thus displayed no obvious increase of survival. The presence of MEK inhibitor had no influence on the levels of surviving cells as well the apoptotic fraction in presence of cisplatin.

To make sure that the observed protective effects were indeed AKT-dependent, mode and consequences of AKT activation were further analyzed. Activation of AKT was observed at Ser473 as well as Thr308. Ser473 is a secondary activation site which is required to be phosphorylated for reaching fully activated AKT. This site can be phosphorylated by various kinases. Besides kinases like DNAPK, ATM or PKCα [184] [185] [186], mTORC2, which comprises the AKT target mTOR, as well as AKT itself were shown to be kinases for Ser473. AKT and mTOR indicate a role for Ser473 as a secondary activation site dependent on primary AKT activity [36] [37]. For Thr308, the only described kinase is PDK1, which is activated by PIP3, the product of PI3K (fig.1; reviewed in [39]). Inhibitor combinations using PD184352 and

LY294002 demonstrated a PI3K-dependent AKT phosphorylation of both phosphorylation sites after MEK inhibition. This demonstrates an activation mechanism mediated via upstream components like e.g. RAS or RTKs. Since high levels of active PI3K/AKT signaling have been frequently linked to an increase in survival and protection from apoptosis (reviewed in [187]), further analyses of the contribution of AKT to the increased survival were conducted. However, since inhibition of PI3K/AKT turned out to strongly reduce the number of living cells in presence of cisplatin, long term experiments in presence of the PI3K inhibitor were not feasible to get further insights into AKT mediated increased survival. Although other groups reported that the inhibition of ERK1/2 signaling went along with increased chemo-sensitivity [174] [181] [182] [183], a protective contribution of MEK inhibition due to a reduced growth rate, and thus reduced rate of DNA synthesis, needed to be ruled out. In addition to the fact that the protective effect was only present in cell lines characterized by concomitant AKT activation, it was addressed whether the increased chemo-resistance is actually AKT dependent and not facilitated by a more direct effect of decreased ERK1/2 activity after MEK inhibition. To this end, AKT activation was achieved by a constitutively active form of AKT3 (myrAKT3) in a MEK independent fashion. Activation of AKT in this manner mimicked the situation observed after MEK inhibition to a high extent as seen on comparable levels of AKT Ser473 phosphorylation. Even more, cells expressing myrAKT3 showed comparable levels of apoptosis protection in presence of cisplatin as seen after MEK inhibition. This proves the direct contribution of activated AKT to the increase in chemo-resistance.

6.2.3 The FOXO target PUMA is a downstream mediator of acquired cisplatin resistance

PI3K/AKT signaling is known to be implicated in cell survival and apoptosis prevention. These functions can be mediated by several distinct mechanisms. Direct phosphorylation of the BCL2 family member BAD or caspase9, both leading to inhibition of apoptosis [188] [189] and the regulation of transcriptionally active downstream machineries are of major importance. Besides NF-κB [190], CREB [191], and MDM2, the latter being implicated in p53 stability [192] [193], the family of FOXO transcription factors is considered as the main apoptosis-regulating factor, that is

targeted by AKT [48]. In our system, MEK inhibition did not only activate AKT but it also led to concomitant phosphorylation of FOXO1 and 3a at sites implicated in cytoplasmatic sequestration and thus FOXO inactivation. The importance of FOXO proteins in AKT mediated survival has already been described earlier [48] [194]. There are also data obtained in human melanoma cell lines, showing that the expression of a FOXO variant that cannot be sequestered in the cytoplasm induces pro-apoptotic genes like *BIM*, *NOXA* or *TRAIL* and repress genes linked to survival (e.g. *BIRC5,* encoding survivin) [137]. This underpins the functional relevance of MEK inhibitor induced AKT and FOXO activation as an important mediator in the prevention of cisplatin induced apoptosis. Similar to MEK inhibition, myrAKT3 led to comparable degrees of FOXO phosphorylation as observed after MEK inhibition.

FOXO transcription factors are not solely involved in apoptosis but are also implicated in proliferation by regulating genes like *CCND1*, *p27* or *p21* [195] [196] [197]. However, since ERK1/2 is known to be an important regulator of cell cycle control and is also implicated in regulation of cell cycle genes, any PD184352-dependent effects on these genes are probably at least partly directly ERK1/2 dependent (reviewed in [198]). The same holds true for apoptosis facilitators like NOXA, BIM or TNF that have been described to be regulated by FOXO [48]. The observed changes in expression of the corresponding genes are likely to be influenced not only by FOXO but also by ERK1/2 [199]. The mRNA and protein levels of the FOXO regulated pro-apoptotic gene *PUMA* [200] [201] which was strongly induced in response to cisplatin, was markedly attenuated in presence of the MEK inhibitor in an AKT-dependent manner. It was, however, not regulated by MEK inhibition in absence of cisplatin. Conversely, Wang et al. found that MEK inhibition can lead to the induction of PUMA [171]. Those results were obtained in cell lines that were driven into apoptosis by MEK inhibitor mono-application, while the cell lines I used were characterized by a very low susceptibility towards MEK inhibitor induced apoptosis. PUMA belongs to the pro-apoptotic BH3 only members of the BCL2 family and is rapidly induced in a p53 dependent manner after DNA damage, e. g. by cisplatin [202] [203]. Mechanistically, PUMA, like the other BH3 only BCL2 members NOXA, BIM, BAD, or BIK is able to induce apoptosis by preventing the pro-survival BCL2 proteins BCL2, BCL-XL or MCL1 from their inhibiting interaction with BAK and BAX. This in turn causes a high permeability of the mitochondrial membrane, resulting in cytochrome c release and thus the execution of the late apoptosis

program (reviewed specifically for melanoma in [204]). PUMA is negatively regulated during the process of melanomagenesis and low levels of PUMA protein are correlated with a poorer survival in melanoma patients [205].

In addition to PD184352, myrAKT3 overexpression also caused an attenuation of cisplatin-induced PUMA induction, clearly demonstrating the AKT dependence of this effect. Furthermore, the functional significance of PUMA in mediating cisplatin induced apoptosis could also be demonstrated, since *PUMA* knockdown enhanced survival in presence of cisplatin. However, other apoptosis effectors might be involved, too.

Taken together, I identified a crosstalk between ERK1/2 and PI3K/AKT/FOXO/PUMA, which mediates increased chemo-resistance towards genotoxic stress in melanoma cell lines. This clearly underscores the importance of a thorough melanoma subtype classification on the molecular level prior to specific therapeutic treatment combinations. Suitable classification markers that predict the described crosstalk mechanism might be found among receptor tyrosine kinases.

6.2.4 RTKs and feedback-regulators in MEK inhibitor induced AKT activation

To adapt to environmental and cellular changes, cells comprise a plethora of signaling crosstalk, integration and regulation, just as the observed activation of AKT signaling after MEK inhibition. A better insight into the mechanisms of such a crosstalk is of high relevance not only with regard to the understanding of basic signaling networks but also in the light of avoiding unwelcome resistance mechanisms. As I demonstrated in my work, these resistance mechanisms might even be of utmost importance in combination therapy.

RTKs and their adaptors, scaffolds and signal transducing complexes are important inducers of ERK1/2 and AKT signaling. As factors associated with RTKs, SOS and GRB1 have been identified to mediate regulatory feedback communication between the two pathways [206]. In addition, different receptors have been described to be implicated in PI3K hyper-activation after RAS/RAF/ERK1/2 pathway inhibition. ERBB3 was shown to be a crucial mediator of MEK inhibitor induced AKT activation in different malignancies [178]. In these cases activation of the receptor was achieved by a MEK inhibitor induced loss of an inhibitory phosphorylation resulting in

relieve of a negative feedback loop. High EGFR activity was identified to be pivotal in rendering colon carcinomas resistant to vemurafenib treatment by activating PI3K [142] [207]. In melanoma, specifically PDGFRß, MET, SRC and IGF1R [117] [118] [119] have been described to be able to confer vemurafenib resistance, each in a specific cellular context. Recently, in a broader approach, the contribution of several active RTKs to resistance against specific kinase inhibition in general was validated in a panel of different tumor types [208]. There again, MET appears to be of high significance for vemurafenib resistance in melanoma, as presence of HGF, the ligand for MET, was able to confer resistance. Interestingly, in co-culture of melanoma cells with different types of stromal cells, paracrine HGF derived from such a stromal niche was sufficient to induce BRAF inhibitor resistance, underscoring again the importance of RTKs in general and MET in particular during those processes [209].

In A375 cells, known to express high levels of EGFR [210], I found that EGF expression was increased after MEK inhibition, indicating the possibility of an autocrine EGFR activation. Since EGFR was identified as a factor involved in resistance mechanisms in colon and gastric carcinomas [207] [211] and in addition to that, constitutes a RTK with a common signaling activity in melanoma [212], we hypothesized that EGFR signaling might contribute to the ERK/1/2- PI3K/AKT pathway crosstalk. Stimulation with EGF of starved A375 as well as LOX IMVI cells slightly enhanced AKT phosphorylation levels after MEK inhibition, implicating a functional role for EGFR. While either MEK inhibition or EGFR inhibition alone did only marginally affect the proliferation rates of LOX IMVI cells, co-application had a strong additive effect. In other words, active EGFR signaling is required for the intrinsic resistance of LOX IMVI cells towards MEK inhibition. In addition, long-term experiments using combinations of cisplatin, MEK inhibitor and the EGFR inhibitor AG1478 demonstrated that the observed increase of survival under genotoxic stress, which is seen in presence of MEK inhibitor, is dependent on EGFR signaling. Since the EGFR inhibitor AG1478 alone did not affect apoptosis or cell growth in these cells, it is very unlikely that the observed effects were due to deleterious features of AG1478 itself. Interestingly, TKI258, a multi-RTK-inhibitor being able to inhibit FLT3, c-KIT, FGFR1/3, VEGFR1/2/3, PDGFRß and CSF-1R with varying kinetics abolished the protective effect of MEK inhibition as well. This indicates that besides EGFR, several other RTKs might be able to participate in the pathway crosstalk. Of note, AG1478 has also been described to inhibit other RTKs like PDGFRß, albeit with a

much lower IC50 value (100nM compared to 3nM). The possible contribution of several RTKs would also be supported by the fact that various RTKs have been identified to be of major importance in $BRAF^{V600E}$ inhibitor resistance in various cell line or tumor settings. Thus, combinations of ERK1/2 pathway inhibitors with different RTK inhibitors carry high potential. In accordance, the application of a MEK inhibitor in combination with imatinib or cyclolignan picropodophyllin (IGF1R inhibitor), chosen to overcome PDGFRß or IGF1R mediated $BRAF^{V600E}$ inhibitor resistance, showed promising results [213] [119]. Similar results were obtained using a FGFR3 inhibitor in a vemurafenib resistant A375 clone that was shown to be dependent on FGFR3 [214]. All these combinations were able to restore the ERK1/2 inhibitor‘s potential to induce growth arrest and apoptosis.

In parallel to the contribution of RTKs in MEK inhibitor induced AKT activation, the role of the Sprouty proteins was analyzed with regard to their potential of activating AKT signaling through feedback mechanisms. SPRY3, which was not differentially regulated after MEK inhibition, was excluded from further analyses. In contrast, SPRED1 and SPRED2 as well as SPRY1, SPRY2 and SPRY4 were strongly downregulated after MEK inhibition. The latter three genes were further investigated, since it is known that SPRY proteins in general are able to negatively regulate RTK signaling upstream of RAS, and therefore they are potentially capable of influencing parallel signal transduction modules like the PI3K/AKT signaling axis [92] [93] [94]. Furthermore, SPRY2 and SPRY4 have been identified to be among the three most downregulated genes after MEK or $BRAF^{V600E}$ inhibition in melanoma [68] Hypothetically, MEK inhibitor induced down-regulation of SPROUTY family members might be responsible for the activation of the AKT pathway. Mechanistically, the absence of a negative feedback-regulator would increase RTK dependent signaling by relieving the inhibitory pressure on RTKs, adaptors or RAS. This in turn could not only positively regulate ERK1/2 signaling output, but also activate parallel RTK or RAS dependent pathways (fig. 26).

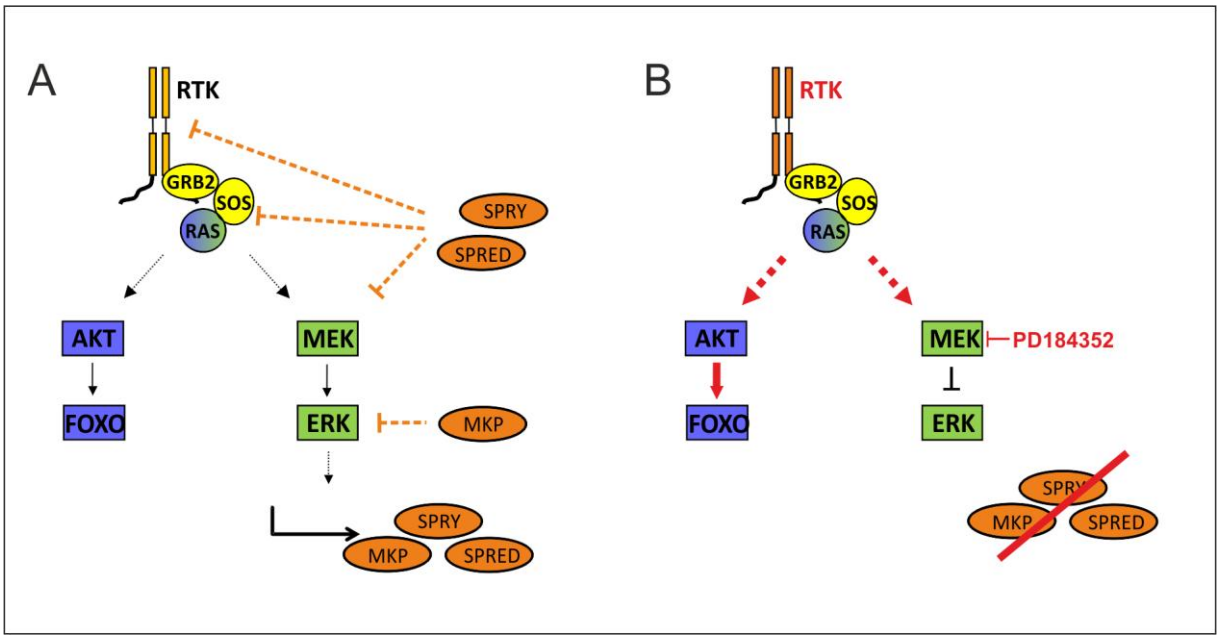

Figure 26: Working model of RTK dependent RAS/PI3K/AKT and RAS/RAF/ERK signaling crosstalk **A:** Without MEK inhibition normal feedback regulation attenuates RTK dependent AKT and ERK signaling. **B:** In presence of MEK inhibitor (PD184352), the ERK dependent feedback is abolished. This leads to increased AKT signaling output which is enhanced by active RTK signaling.

Despite the considerable level of regulation on the mRNA level, the amount of SPRY1 and SPRY2 protein seemed to be only slightly altered after MEK inhibition. Solely SPRY4 protein levels paralleled the strong reduction as observed at the transcript level. All three proteins, however, were strongly upregulated in melanoma cells in comparison to primary human melanocytes demonstrating the transcriptional dependence on active $BRAF^{V600E}$.

Functional analysis of SPRY1, 2 and 4 using siRNAs showed no effect in the tested melanoma cell lines. No change in ERK1/2 or AKT phosphorylation could be observed. Consequently, when challenged with cisplatin, no difference in cleaved caspase3 induction or survival could be detected. The failure to induce a change in ERK1/2 or AKT phosphorylation might be either due to insufficient knockdown efficiency or due to compensatory mechanisms of other SPROUTY family members coping with the loss of a single one. Thus, a broader approach in targeting SPRY functions was achieved by using dominant negative versions of SPRY2 (SPRY2dn) and SPRY4 (SPRY4dn). The dominant negative function of either one was described to be able to interfere with both, wildtype SPRY2 and SPRY4 [133]. Both dominant negative proteins were expressed at comparable levels, but only expression of SPRY2dn, either alone or in combination with siRNA against *SPRY1* and *SPRY4*, led to enhanced proliferation and a slightly increased ratio of surviving cells in presence of cisplatin. Still, the effect was rather weak. An explanation for this might be the

existence of compensatory mechanisms mediated by residual SPRY proteins as well as SPRED proteins. Redundant functions of SPRY family members have already been described in embryogenesis [76]. To make a clear statement about the involvement of SPRY proteins in MEK inhibitor induced feedback, the parallel inhibition of all SPRY genes will have to be optimized. Furthermore, a contribution of SPRED1 or SPRED2 to the feedback loop may be possible. With the identification of SPRED1 loss to be causative for the Legius syndrome which is characterized by the presence of *Café au lait* spots, a prominent role for SPRED1 in melanocyte biology was identified previously [215]. Furthermore, it has been shown mechanistically that SPRED1 is able to regulate signaling at the level of RAS or upstream. In Legius syndrome, mutated SPRED1 is no longer able to interact with neurofibromin, a negative regulator of RAS. This leads to elevates RAS-GTP levels and thus elevated RAS signaling [99]. Additionally, SPRED1 was described to interact with FGFRL1, a member of the FGF receptor family positioned upstream of RAS [216]. Unpublished, preliminary experiments show that a dominant negative version of SPRED1 might have an effect on ERK1/2 and AKT phosphorylation levels.

6.3 Perspectives

The observed treatment induced AKT activation is of central importance for cellular survival. AKT mediated suppression of pro-apoptotic factors like PUMA is able to prevent beneficial effects of genotoxic melanoma therapy. Thus, combined inhibition of AKT or RTKs and the MAPK signaling pathway might be a promising strategy, even in the light of an additional genotoxic therapy. Taken together, the presented data underscore the relevance of a thorough classification of melanomas to identify a suitable treatment regimen.

7 Bibliography

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